

The Effect of Probiotics on Human Gastrointestinal Microbial Communities

by

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Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biology

Program

YOUNGSTOWN STATE UNIVERSITY

August, 2015

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Abstract

The human microbiome is home to roughly 100 trillion microbial, which have been shown to assist in various host physiological functions. Research shows that probiotic consumption may benefit host health. Therefore this is a two part study: project I is a study determining the effects of yogurt (probiotic) consumption on GI microbial structure and diversity, and project II is a study examining the effects of probiotic pill consumption has on stress response (cortisol) for healthy human test subjects exposed to a stressor.

In chapter II we used terminal restriction fragment length polymorphism, Sanger sequencing, and qPCR to characterize microbial communities of the GI tract. Non-metric multidimensional scaling analyses, Shannon Wiener diversity index values, and qPCR (targeting *Lactobacillus*) analysis showed a temporal influence on microbial community, diversity and absolute abundance of *Lactobacillus* on human test subjects that consumed 250g of yogurt per day for 42 consecutive days.

In chapter III, we quantitated *Lactobacillus* using qPCR and determined stress response (via cortisol) using a salivary enzyme immune assay (EIA). Results from this study showed a decrease in stress response and an increase in the relative abundance of *Lactobacillus* in individuals that consumed one probiotic pill per day for 30 consecutive days. In comparison, the control group showed no changes in stress response or *Lactobacillus*.

Results from this study suggest the potential for probiotics as therapeutic treatment for host physiological function and stress response. However, further research is necessary to determine the effects probiotics have on human test subject.

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Acknowledgments

I first would like to thank my advisor, Dr. Carl Johnston, for his guidance and support. Working in his lab truly sparked my interest in research and allowed me to find my passion in the gut microbiome. I am forever grateful in all of the opportunities that he provided me and for all of his advice when writing papers and scientific media.

I'd like to thank my committee members, Dr. Fagan and Dr. Min, for critiquing my thesis as well as providing guidance in my graduate career. I'd like to thank current and former members of Dr. Johnston's lab: Justin Waldern, Rachel Centifonti, Aaron Stevens, and Jennifer Moore. I also would like to thank Dr. Thomas Diggins for providing statistical advice and Ed Budde for providing technical support and training on qPCR. and Michael Bowman for allowing me to use the elevated high ropes located at Youngstown State University's Andrews Student Recreation and Wellness Center.

I would like to thank Dr. Patricia Johnston for mentoring me and providing analytical advice. Working with her pushed me to be a better researcher and I am appreciative of all the advice she has provided to me. Because of you I believe that I am better write in comparison to when I first worked in Dr. Johnston's lab.

I would like to thank my parents, Stephen and Virginia Lisko, for allowing me to pursue my dream of receiving a higher education in Biology and believing in me. Without them I would not be the person I am today.

Finally, I would like to thank the Youngstown State University Biology Department, Graduate Studies, STEM, and the YSU Foundation for providing funding throughout my graduate career.

Prologue

This thesis is organized to produce initial drafts of 2 publications, i.e., Chapter II and Chapter III will be submitted as 2 independent publications once research results are fully incorporated. Chapter I provides general background on the human gut microbiome. Chapter II is a study showing the effect long-term yogurt consumption has on GI microbial structure and diversity. Chapter III is a study examining the effects probiotic pill consumption has on stress response (cortisol) for healthy human test subjects that are exposed to stressor. Finally, chapter IV describes the future work for both studies.

Chapter I: Introduction

1.1 The human gut microbiome

In the past, research of microorganisms and human health has focused primarily on the negative impact pathogens have on the human body. Researchers have begun to investigate the microbial communities of human host and how they can have a beneficial effect on health.

The term gut microbiome (also known as microbiota) is used to describe the ecological community of commensal and symbiotic microbes that reside in the gastrointestinal tract (GI) and all of their interacting genomes (Kinross et al., 2011). The human microbiome is estimated to be composed of nearly 100 trillion microbial cells that outnumber our human cells 10 to 1 (Hamady & Knight, 2009; Turnbaugh et al., 2013). Many of the present microbiota have been shown to colonize virtually every surface of the human body that is exposed to the external environment, with the most heavily colonized organ being the GI tract (Sekirot, et al., 2010; Dominguez-Bello et al., 2010). The microbiota of the GI tract is not considered to be homogenous. Both cell density and microbial diversity increase as one moves further down the GI tract toward the colon,

which contains roughly 10^{11} - 10^{12} bacteria cells per gram of luminal content (Ratsall, 2004).

Although the human gut is home to nearly 50 bacterial phyla, it is dominated primarily by *Bacteroidetes* and *Firmicutes*, whereas *Proteobacteria*, *Verrumicrobia*, *Acintobacteria*, *Fusobacteria*, and *Cyanobacteria* are present in minute proportions (Sekirov, et al., 2010). Furthermore, some of the most dominant groups of bacteria of the human GI tract include *Bifidobacteria*, *Lactobacillus*, *Streptococcus*, and *Clostridia* (Hamady et al, 2009). It should be noted that limitations in fecal collection, DNA extraction, and molecular analysis can result in an underestimation of microbial groups/phyla.

Development of the microbiome

The human microbiome is first inoculated immediately after passage through the birth canal (Koenig et al., 2011). Initially, the infant microbiome is moderately low in diversity. However, as the infant continues to progress in age, so does the abundance of major bacterial taxonomic groups. Some of the earliest colonizers are typically aerobic organisms (e.g. *Staphylococcus*, *Streptococcus*, and *Enterobacteria*) whereas later organisms tend to be anaerobes (*Eubacteria* and *Clostridia*) (Palmer et al., 2007). Koenig and colleagues (2011) documented increased diversity over time in relation to a gradual change in community composition. Furthermore, Koenig showed that these gradual changes are in relation to the effect of typical life events of a growing infant (changes in diet or health, and use of antibiotics). After one year of life, the GI community, although distinct, has a convergence towards a microbial community similar to that of an adult

(García-Albiach et al., 2008). Many of the microbes that establish in the GI tract provide a range of beneficial functions for the human host, such as protection against pathogens, development of the immune system, as well as influencing basic physiological tasks such as digestion (Cho & Blaser 2012; DiBaise et al. 2008; Turnbaugh et al. 2013).

Immunomodulation of GI tract

The immune system recognizes, responds to, and adapts to various pathogenic infections (Round & Mazmanian, 2009). Research utilizing Germ Free (GF) mice, which lack microbiota, have demonstrated the importance of gut microbiota on host immune health. For example, studies using GF mice have demonstrated a reduction in secreted immunoglobulin A and G, which are antibodies that protect the body from various infections. (Sekirov et al., 2010).

Commensal micorbiota, such as *Bifidobacterium* and *Lactobacillus* (both are also probiotics, microorganisms consumed for their potential health benefits), have been shown to play an important role in host immunity. *Bifidobacteria* exhibit the ability to subdue the toxic effects of Shiga toxin (STX), which is produced by *Enterohaemorrhagic Escherichia coli* (EHEC) O157:H7 (Asahara et al., 2004 and Fukuda et al. 2011). Fukuda and colleagues (2011) showed that germ free (GF) mice that were fed *E. coli* O157:H7 died within 7 days. However, if germ free mice were inoculated with *Bifidobacterium longum* subsp. *longum* JCM 1217, they survived. Furthermore, Fukuda showed that these effects could be attributed to increased production of acetate, which may promote the defense functions of epithelial cells, thus protecting the host against lethal infection. A similar study inoculating GF mice with a combination of *Lactobacillus reuteri*

(commensal microbe of the GI tract) and EHEC showed that *L. reuteri* was able to suppress the colonization of EHEC and significantly protect from the manifestation of disease, when compared to GF mice inoculated with only EHEC (Eaton et al., 2011). Although, these microbes are a small representation of the vast ensemble of microbiota that inhabits the GI tract, they still demonstrate the importance the GI microbiota in assisting the host in immune defense. The inoculation with GF mice also suggests the possibility of alternative therapeutic approach for fighting various infections.

Disruption of gut microbiota

Although the gut microbiome is fairly stable throughout our lives, various factors can have detrimental effects on the microbiome composition. Diet, age, and antibiotic usage can have both quantitative and qualitative effects on the microbiota (García-Albiach et al., 2008). For example, the use of antibiotics, which are commonly administered by clinical physicians, have been shown to reduce and alter bacterial population densities (Preidis & Versalovic, 2009). This reduction in microbial communities has been shown to cause long-term disturbances for select individuals (Jernberg, et al., 2007). Antibiotics can reduce commensal microbiota that inhabit our GI tracts and allow for the overgrowth of opportunistic pathogens, such as *Clostridium difficile*. (Jernberg, et al., 2007; Bartlett et al., 2002). Chang and colleagues (2008) were able to demonstrate by non-culturing techniques (i.e. molecular techniques), that recurrent *C. difficile* associated diarrhea (CDAD) was associated with lower microbial diversity when compared to control subjects (no signs of CDAD).

Microbiota and Obesity

Recent studies provide evidence of obesity being associated with structural alterations in the GI microbiota and suggest the possibility for therapeutic manipulation of these microbes. One such study shows obesity to be associated with large-scale shifts in the dominant phyla of bacteria in both human and animal models (Ley 2010). The GI microbial communities of adult human monozygotic and dizygotic twins were evaluated to determine what correlations exist with obesity or leanness in terms of microbial community composition. The results showed that a diverse microbial community could yield a core microbiome at a functional level (e.g. a community of microbes that together have particular pathways of carbohydrate metabolism), while deviations from this core can be associated with different physiological states such as obesity (Turnbaugh et al., 2008). Furthermore, microbial community gene content, as displayed in *murine* models, showed that an obese associated microbiome is enriched with genes that are capable of importing and metabolizing otherwise indigestible polysaccharides, which are then stored as adipose tissue. (Turnbaugh et al., 2012). Kadooka et al. (2010) were able to demonstrate the ability of the probiotic bacteria *Lactobacillus gasseri* to reduce adiposity and body weight in human test subjects, demonstrating potential benefits on metabolic disorders associated with obesity.

Probiotics

Previous research suggests that the microbiome is malleable and changes in the microbiome have consequences for human health. However, many therapeutic approaches targeting the microbiome may help counteract these negative effects. One

such positive manipulation is the use of probiotics, which are defined as “live microorganisms that when administered in adequate amounts confer a health benefit on the host” (Uyeno, et al., 2008). Over the past decade, probiotics have generated a vast interest as a potential new method of prevention for multiple gastrointestinal diseases, as well as having an effect on the immunological response (Preidis & Versalovic, 2009). Sonnenburg and colleagues (2006) colonized germ-free rodents with *Bacteroides thetaiotaomicron* (a common commensal gut microbe) and *Bifidobacterium longum* (a common probiotic bacterium). Simultaneous whole genome transcriptional profiling of both species as well as analysis of the GI-associated carbohydrates revealed that the presence of *B. longum* stimulated an increase in polysaccharide degradation, thus demonstrating the effect probiotic bacteria can have on metabolic function (Sonnenburg, et al., 2006). Gao et al. (2010) showed the effect of probiotic dosage on antibiotic associated diarrhea (AAD) and CDAD after patients were administered antibiotics. Individuals who received probiotics, more specifically at higher doses, were less likely to have CDAD infection (Gao et al. 2010).

Yogurt

Although, in the past decade there is increased attention on viewing ourselves as microbial ecosystems and the knowledgeable benefits of probiotics, there is still not enough information about the dynamics of microbial communities on individuals who consume yogurt regularly (Maukonen et al 2008). One study compared the effects of lactobacilli-containing yogurt and non-probiotic containing yogurt on the composition of gut microbiota of healthy individuals who ingested a daily serving for 20 days. Utilizing

sequence-specific small subunit (SSU) rRNA cleavage method, with probes specific for the detection of common gut microbiota, they were able to determine that there was indeed a change in microbiota resulting from digestion of yogurt, primarily in two groups of bacteria (*Bacteroides* and *Prevotella*., and the *C.coccoides*-*E. rectalei* group). However, the change did not seem to depend upon whether there were probiotic bacteria present (Uyeno et al., 2008). Another study compared the effect of the antibiotic clindamycin on intestinal microflora in subjects ingesting yogurt with probiotic microorganisms (*Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Lactobacillus* F19) compared to subjects ingesting placebo yogurt. Based on culture techniques they were only able to conclude there to be no effect on *Bacteroides fragilis* of the GI tract (Sullivan et al., 2003). However, it should be noted that these studies never used broad molecular techniques, which allows for the analysis of unculturable microorganisms.

Chapter 2: A Dietary Effect of Yogurt Consumption on GI Microbial Communities and Microbial Community Diversity of Healthy Individuals

Introduction

Although there is increased attention on the benefits that probiotic therapy may have on metabolic function and infections, there is still sparse information on the dynamics of microbial communities of individuals who consume commercially available multi-species probiotics. The aims of this study were to determine microbial community structure after a specific dosage of yogurt was administered to healthy human subjects. Terminal restriction fragment length polymorphism (T-RFLP) (Liu et al., 1997) was used in this study to determine microbial community composition. T-RFLPs was chosen

because it is a one of the most commonly used high-throughput fingerprinting methods that is relatively affordable (Schüte et al., 2008). We hypothesized that introducing a yogurt diet to human test subjects will: i) increase the abundance of probiotic bacteria, ii) alter the microbial community structure, and iii) increase GI microbial diversity.

Methods

Enumeration and confirmation of Probiotic Bacteria

Several commercially available brands of yogurt were selected from a local grocery store for this study. To test for their viability, 1g of each yogurt was added to 99 mL of sterile water (10^{-2}) and a ten-fold serial dilution was carried out to 10^{-8} . A total of 100 µl of the dilutions, ranging from 10^{-6} - 10^{-8} , were used to inoculate agarose plates that enrich for *Lactobacillus* (LBS Agar) and *Bifidobacterium* (BSM Agar). For a control, a ten-fold serial dilution was performed on 1 mL of sterilized water. A total of 100 µl of diluted controls, ranging from 10^{-6} - 10^{-8} , was used to inoculate LBS and BSM agarose plates. Inoculated plates were incubated anaerobically using GazPak Pouch System (BD technologies, Franklin Lakes, NJ USA) at 37°C for 48-72 hours. After bacterial growth was observed, colonies were enumerated and colony forming units (CFUs) calculated. Yogurt samples with the best CFU counts were then selected for the study in trial 1 and 2 as described below.

Trial 1

Three healthy health test subjects over the age of 18 were selected for this study. All participants had no known lactose intolerance and had consumed some form of

probiotics in the past (i.e. yogurt, probiotic pills, etc.). Subjects were labeled alphabetically (A, B, and C) for the purpose of anonymity. To create a baseline (Day 0), test subjects were required to abstain from the consuming any form of probiotics or antibiotics while maintaining their regular diet for two weeks. After creating the baseline, individuals were then required to consume 450g of yogurt per day for 10 consecutive days while maintaining their regular eating habits. All volunteers gave written informed consent that was approved by the Institutional Review Board (IRB) at Youngstown State University.

Sample collection

Fresh fecal samples were collected on Day 0, Day 5 and Day 10 of the study. Each individual collected their own fecal samples in a sterile 90 mL container (Therapak Co., Buford, Georgia USA), which was sealed, placed on ice, and immediately delivered to the lab to be stored at -20°C until further analysis.

Extraction of Fecal Microbial DNA

Microbial DNA was isolated from 180-220 mg of feces using the Powersoil DNA Isolation Kit (Mobio, Carlsbad, CA USA) following manufactures instructions with minor modifications. Purified DNA was then stored at - 20°C until further analysis.

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

A polymerase chain reaction was used to amplify 16s rRNA genes using primers 8F (5'- AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-

GGCTACCTTGCCACGACTTC-3') (Zhang et al., 2008). The 8F primer was 5' labeled with 6-carboxy-flourescein phosphoramidite (FAM). Each 25 µl PCR reaction contained: 0.5 µl of each primer (10mM), 12.5 µl Go Taq Green Master Mix 1X (Promega, Madison, WI USA), 2 µl of DNA, and 9.5 µl of molecular grade water. The following conditions were used for PCR amplification: an initial denaturing step of 94°C for 3 minutes, 30 cycles of denaturing at 94°C for 30 seconds, annealing for 55°C for 30 seconds, and elongation for 72°C for 90 seconds, followed by an elongation step of 72°C for 7 minutes. PCR products were visualized by gel electrophoresis to verify proper amplification size and length of the amplified fragment. PCR products were then digested using HaeIII and incubating at 37°C for 16 hours. After digestion, samples were then purified using Wizard® SV Gel and PCR Clean-Up system (Promega, Madison, WI USA) according to manufactures instructions.

Digested samples were then sent to the Ohio State University Plant Microbe and Genomics Facility (Columbus, OH USA) for fragment analysis using a 3730 DNA Analyzer (Applied Biosystems, Inc. Waltham, MA USA) using a LIZ1200 size standard and minimum peak height of 50 fluorescence units.

Trial 2

Six healthy test subjects over the age of 18 were selected for this study. All participants who consumed yogurt were had no prior history of lactose intolerance and must have consumed some form of probiotics in the past. Test subjects were assigned letters A, B, C, D, F, and G to keep anonymity. Test subject D was chosen a control subject since that individual had not consumed yogurt for several years, and did not

consume yogurt throughout the entire study. To create a baseline (Day 0), test subjects refrained from consuming any form of probiotics or antibiotics for one week. After a baseline was established, individuals consumed 250g of commercially available yogurt per day for 42 consecutive days. Each participant was to provide fecal samples on Day 0, 7, 14, 21, 28, 35, and 42. All volunteers gave written informed consent that was approved by the Institutional Review Board (IRB) at Youngstown State University.

DNA Extraction

Microbial DNA was extracted from 180-220 mg of fecal material according to QIAamp DNA Stool Isolation Kit (Qiagen, Valencia, CA, USA) protocol. Purified DNA was then stored at - 20°C until further analysis. T-RFLP analysis was then performed on all microbial DNA that was isolated as described above.

DNA Preparation for Sanger Sequencing

Due to budget constraints, isolated microbial DNA from test subjects B, C, and D at Days 0 and 42 were selected for further molecular. Subjects B and C were chosen since they showed a change in microbial community composition from yogurt consumption based on T-RFLP data, and test subject D was chosen because they were the control.

Microbial DNA isolated from fecal samples B, C and D for each sampling day (Day 0 and 42) was used to amplify bacterial 16s rRNA genes. *Lactobacillus* DNA was amplified using primers Lac1 (5'-AGCAGTAGGGAATCTTCCA-3') and Lac2 (5'-ATTYCACCGCTACACATG-3') (Walter et al., 2001). *Bifidobacterium* was amplified using primers G-Bifid-F (5'-CTCCTGGAAACGGGTGG-3') and G-Bifid-R (5'-

CGTGTCTTCCCGATATCTACA-3') (Matsuki et al., 2004). Each PCR reaction (*Bifidobacteria* and *Lactobacillus*) contained: 0.2 μ M of each primer, 10 μ l of Go Taq Master Mix 1x, 2 μ l of DNA, and 7.2 μ l of molecular grade water. Samples were amplified using Px2 Thermocycler (Thermoscientific, Waltham, MA, USA), the thermocycler settings for PCR amplification of *Lactobacillus* and *Bifidobacteria* can be found in Table 1. PCR products were then purified using Wizard® SV and PCR Clean-Up System (Promega, Madison, WI, USA) according to manufacturer's protocol. Clean samples were then frozen at -20 °C until further analysis.

Clone Library

Cleaned PCR products were cloned into competent *Escherichia coli* cells using the Strataclone PCR cloning Kit (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's protocol. Two sets of dilutions, pUC18 control plasmids (provided by kit), and provided StrataClone SoloPack competent controls were plated on LB medium, containing 0.1 mg/ml of ampicillin and 4 μ l of X-gal. Plated samples were then incubated over night at 37°C. White recombinants were then transferred to a 96-well plate containing LB medium, 0.1 mg/ml of ampicillin, and 10 % glycerol. Transferred samples were then incubated at 37°C overnight while shaken at 300 rpm. The insert size of the colony was then determined by PCR amplification as described for *Lactobacillus* and *Bifidobacteria*. We were able to isolate a total of 520 clones (273 from *Lactobacillus* and 243 for *Bifidobacteria*), which were sequenced at the University of Kentucky Advanced Genetic Technologies Center, Lexington, Kentucky.

Table 2-1 Thermocycler settings and bp length for primers used in this study.

Target	Primers	Initial Denaturing	Denaturing	Annealing	Extension	Final Extension	bp length	cited
<i>Bifidobacterium</i>	G-Bifid-F G-Bifid-R	95°C for 5 min	95°C for 30 s	55°C for 20 s	72°C for 30 s	72°C for 5 min	549-563	Matsuki et al. 2002
30 cycles								
<i>Lactobacillus</i>	Lac 1 Lac 2	94°C for 3 min	94°C for 30 s	53°C for 1 min	68°C for 1 min	68°C for 7 min	380	Water et al. 2001
35 cycles								
Universal Bacteria	8F 1492R	94°C for 3 min	94°C for 30 s	53°C for 1 min	72°C for 1 min 30 s	72°C for 7 min		Zhang et al. 2008
35 cycles								

Phylogenetic Analyses of GI Microbial Communities

Bifidobacterium and *Lactobacillus* 16s rRNA gene sequences were first checked for quality using Vector NTI Software (Life Technologies Corp., NY, USA) and manually selected and organized. Sequences were aligned using Clustal W and evolutionary distance was constructed by neighbor-joining method (Saitou and Nei, 1987). A phylogenetic tree was constructed using MEGA (Molecular Evolutionary Genetics Analysis; <http://www.megasoftware.net/index.html>) software, version 5.0 (Kumar et al., 2004). Bootstrap resampling analysis of 100 replicates was performed when constructing the phylogenetic tree to estimate the confidence of tree topology.

Quantitative PCR analysis of Lactobacillus 16s rRNA genes

To quantify *Lactobacillus* genes, quantitative PCR (qPCR) was performed using primers Lac1 and Lac2. Standard curves were constructed from isolated *Lactobacillus* DNA from probiotic pills grown on selective media (LBS). Standards constructed by performing a serial dilution of 10^{-3} - 10^{-9} of *Lactobacillus* DNA.

Real-time PCR was performed using the iQTM5 real-time detection system (Bio-Rad Inc., Hercules, CA, USA). The qPCR reaction was performed using a total volume of 25 µl of the following reagents: 12.5 µl of SYBR Green buffer (Qiagen, Valencia, CA, USA), 9.0 µl of RNase free water, 25 µM of primer, and 2 µl of DNA. The reaction conditions for amplification were 95°C for 3 min, 40 cycles of 95°C for 10s and 55°C for 30s.

Statistical Analysis

Nonmetric multidimensional scaling (NMDS) was used to compare T-RFLP fragments of gut microbial DNA from different subjects at various time points. NMDS was chosen because it is an ordination technique that doesn't require linear variables (as in principles-component analysis). There is also no need for specific distance measures (covariance), and it makes few assumptions about the data (Holland, 2008). Statistical analysis performed using SPSS 12.0 for windows.

The Shannon Wiener diversity index was calculated using restriction fragments (T-RFs) to analyze the microbial diversity for each test subject at Days 0, 7, 14, 21, 28, 35, and 42.

Results

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis for GI microbiome variation and diversity

A total of 61 bacterial terminal restriction fragments (T-RFs), ranging from 59 to 438 bp in length, were detected from collected fecal samples (Table 2-2, Fig. 2-1). On average, 25 T-RFs were recovered for test subject A, 32 T-RFs for test subject B, 33 T-RFs for test subject C, 28 T-RFs for test subject D, 26 T-RFs for test subject F, and 24 T-RFs for test subject G (Table 2-2).

Analysis of T-RFLP profiles by nonmetric multidimensional scaling (NMDS) differentiated the GI microbial samples according to sampling time (Fig. 2-2). T-RFs at Day 0 were the furthest away from the center of the axis for test subjects A, B, and F, but they begin to differentiate at Day 7. By Day 14 of yogurt consumption, microbial

communities begin to cluster together, indicating microbial communities of similar composition (Fig. 2-2). Test subject D (control) also differentiated over time, but not as drastically as the experimental group (Fig. 2-2). Test subject A begins to cluster toward the center of the axis, but at Day 42 it migrates away from the cluster (Fig. 2-2).

Shannon Wiener diversity index analysis (Fig. 3; Table 3) showed the highest index value (H') for test subject C for all time points except for Day 28. Index values also showed an increase in microbial diversity from Day 0 to Day 14. However, after Day 14 microbial diversity appears to plateau and stay in a state of flux throughout the remainder of the study (Fig. 3; Table 3). Although, it should be noted that all test subjects had higher index values at each time point, when compared to Day 0, except for test subjects A and D (control subject) (Fig. 3; Table 3). Test subjects G and F had the lowest index values at Day 0 in comparison to all other test subjects. Test subjects G and F also showed the greatest increased index values for all time points, when compared to Day 0 (Fig. 3; Table 3).

Table 2-2 Number of T-RFs for human test subjects at Day 0 to Day 42 and average number of T-RFs for all human test subjects in this study.

Day	A	B	C	D	F	G
0	24	28	28	26	24	23
7	30	32	35	30	21	24
14	25	30	39	31	33	21
21	24	30	29	27	23	28
28	26	40	36	27	28	28
35	19	30	31	25	25	22
42	30	34	32	29	25	24
Average T-RFs	25	32	33	28	26	24

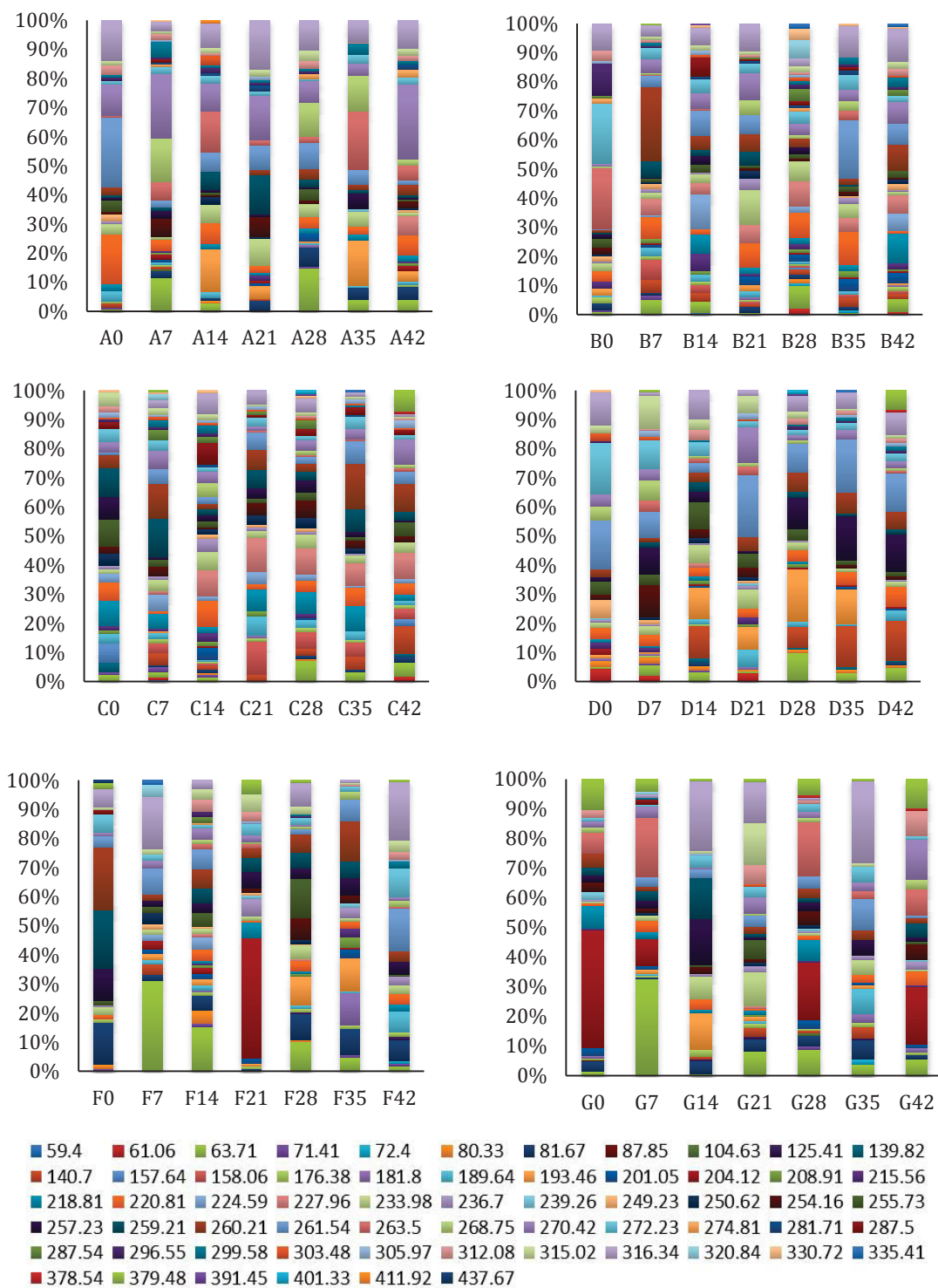


Fig. 2-1 GI bacterial community composition from test subjects A, B, C, F, G, and D (control) at times 0, 7, 14, 21, 28, 35, and 42. The figures show relative abundance of terminal restriction fragments (T-RFs) that contribute to the community with more than 2 %.

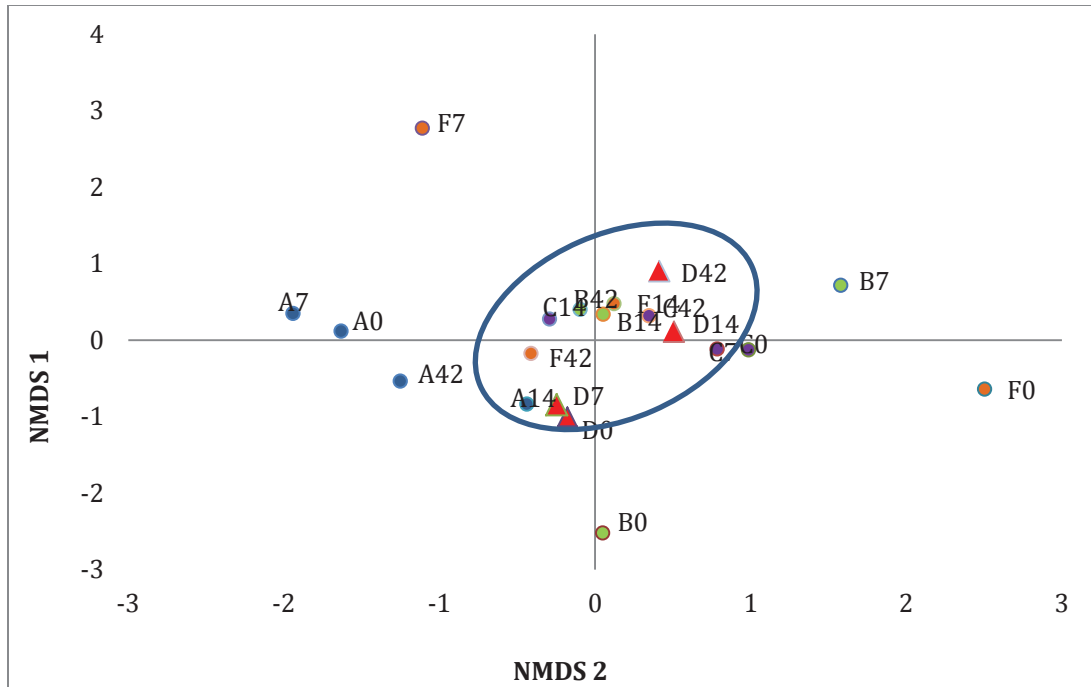


Fig. 2-2 NMDS ordination showing similarity and distribution of microbial communities (T-RFs of the 16s rRNA gene) for test subjects A, B, C, D, and F at times 0,7,14, and 42. Each color represents the microbial community from and individual test subject. Circles = Experimental Group; Triangles = Control.

Table 2-3 Diversity indexes based on the number of terminal restriction fragments (T-RFs) recovered from test subjects A, B, C, D, F, and G at Days 0, 7, 14, 21, 28, and 45. Bold values indicates highest index values at each time point.

Day	A	B	C	D	F	G
0	2.49	2.63	3.06	2.77	2.25	2.32
7	2.72	2.86	3.18	2.98	2.44	2.36
14	2.78	3.11	3.33	3.07	3.18	2.39
21	2.64	3.00	3.02	2.80	2.35	2.86
28	2.84	3.38	3.32	2.83	2.87	2.71
35	2.56	2.91	3.00	2.48	2.83	2.60
42	2.38	3.11	3.13	2.99	2.73	2.67

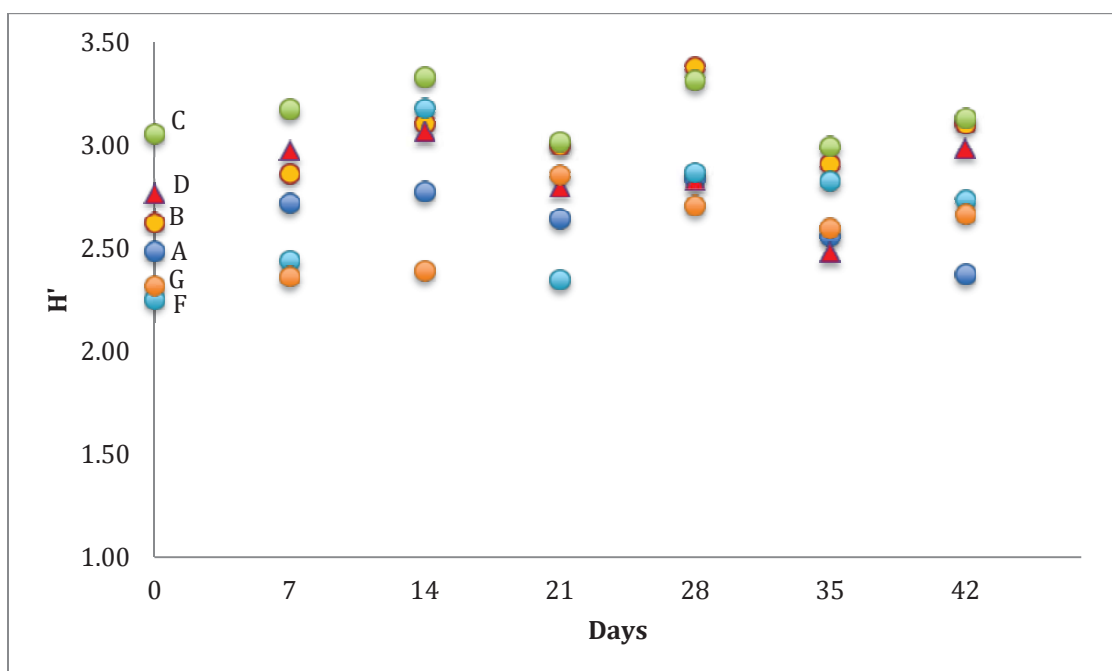


Fig. 2-3 Shannon diversity index (H') values showing variances in diversity for test subjects A, B, C, F, G, and D (control) at Days 0, 7, 14, 21, 28, 35, and 42.

Bacterial Taxa

Sequences from a 282 *Bifidobacteria* and 272 *Lactobacillus* bacterial clones were analyzed for quality using Vector NTI Software (Life Technologies Corp., NY, USA) for test subjects B, C and D (control) at Days 0 and 42. A total of 263 *Bifidobacterium* (67 for B, 120 for C, and 76 for D) gene sequences and 146 *Lactobacillus* (48 for B, 39 for C, and 59 for D) gene sequences were analyzed. Recovered sequences were matched to sequences found using BLASTn with the highest score values.

Clone libraries constructed of *Bifidobacterium* at the species level for sample B0 was composed of *Bifidobacterium longum* (19%) and *Bifidobacterium pseudocaenulatum* (81%) and sample B6 was composed primarily of *B. longum*. Sample C0 consisted of *B. longum* (21%), *Bifidobacterium bifidum* (44%), *Bifidobacterium adolescentis* (24%), and *B. pseudocatenulatum* (10%) and sample C6 contained *Alloscardovia omnicolens* (4%), *B. longum* (16%), *B. bifidum* (12%) and *B. adolescentis* (58%). Sample D0 was made up of *B. longum* (47%), *B. adolescentis* (44%) and *B. pseudocatenulatum* (9%) while sample D6 consisted of *B. bifidum* (18%), *B. adolescentis* (36%), and *B. pseudocatenulatum* (45%).

Clone libraries constructed from PCR products using species specific primers for *Lactobacillus* for sample B0 was composed *Lactobacillus iners* (27%) and *Lactobacillus casei* (73%). Sample B6 consisted of *L. casei* (78%) and *Lactobacillus rhamnosus* (18%). Sample C0 consisted of *Lactobacillus gassrei* (45%), *Lactobacillus crispatus* (20%), *L. casei* (10%), *Lactobacillus ruminis* (5%), and *Pediococcus stilesii* (20%). Sample C6 contained *L. casei* (32%), *L. rhamnosus* (32%), *L. ruminis* (26%) and *Lactobacillus reutrei* (11%). Sample D0 contained *L. gasseri* (8%) , *L. crispatus* (4%), *Lactobacillus*

sakei (4%), *L. casei* (16%), *L. rhamnosus* (28%), *Dolosigranulum pigrum* (4%), *Granulicatella adiacens* (4%), *L. reuteri* (4%), *Pedicoccus stilesii* (24%), and *Lactobacillus plantarum* (8%). Sample D6 contained *L. gasseri* (58%), *Lactobacillus elbruckeii* subsp. *Bulgaricus* (9%) *Lactobacillus reuteri* (21%), *Weissella paramesenteroides* (3%), *Lactobacillus homohiochii* (6%).

Quantitative PCR of Lactobacillus 16s rRNA genes

Absolute quantification of *Lactobacillus* 16s rRNA by qPCR (Fig. 2-6) revealed that test subject B at Day 0 had 6.87e-05 μ M *Lactobacillus*, which increased to 7.82e-01 μ M at Day 42, indicating an increase in *Lactobacillus* bacteria. Interestingly, test subject C showed a slight decrease in *Lactobacillus* 16s rRNA gene content from Day 0 (2.33e-01 μ M) to Day 42 (5.65e-02 μ M). Control subject D at Day 0 had 6.49e-04 μ M of *Lactobacillus* genes and at Day 6 2.42e-04 μ M.

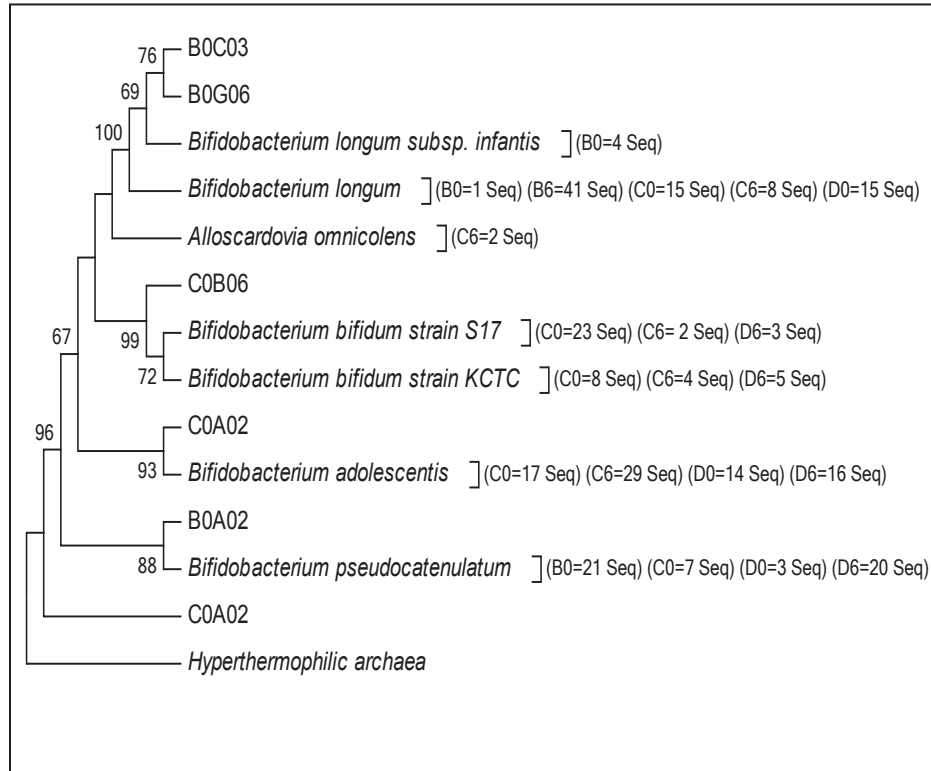


Fig. 2-4 Phylogenetic tree of *Bifidobacterium* 16S rRNA gene sequences retrieved from test subjects B, C, and D (control) fecal material. The tree was constructed using the neighbor-joining method. Bootstrap values (in %) are based on 100 replicates each (distance and minimal evolution) and are shown at nodes with >50% support.

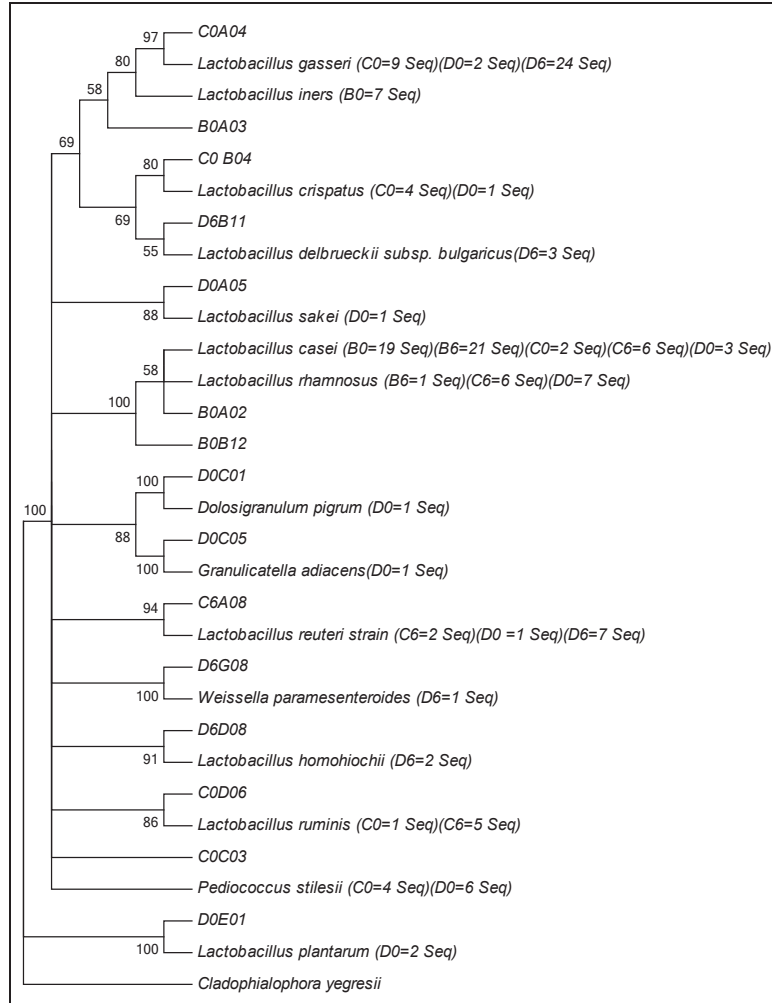


Fig. 2-5 Phylogenetic tree of *Lactobacillus* 16S rRNA gene sequences retrieved from test subjects B, C, and D (control) fecal material. The tree was constructed using the neighbor-joining method. Bootstrap values (in %) are based on 100 replicates each (distance and minimal evolution) and are shown at nodes with >50% support.

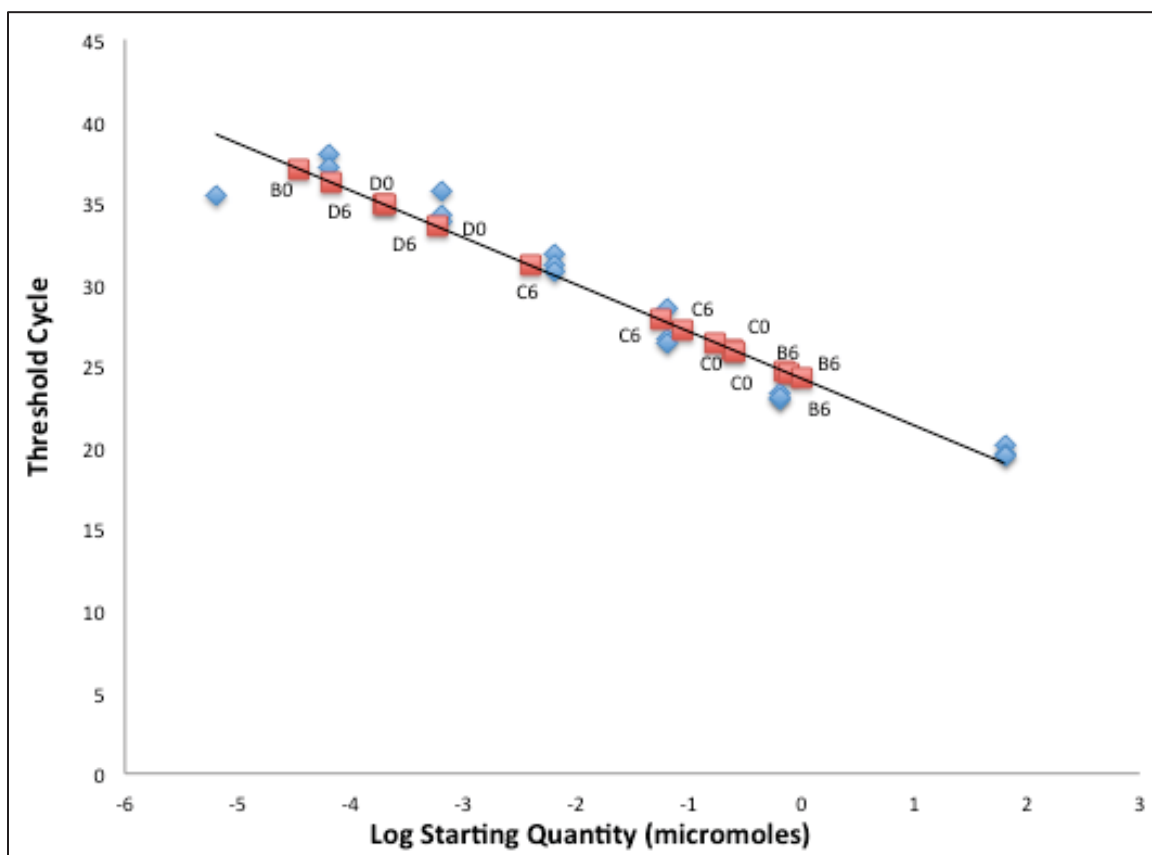


Figure 2-6: Quantitative PCR for *Lactobacillus* detected from human subjects B, C, and D at Days 0 and 42. Standard curve (blue diamonds) was from *Lactobacillus* DNA.

Discussion

In this study, we examined the temporal changes of GI microbial community composition and diversity of healthy human test subjects that consumed 250g of yogurt per day for 42 consecutive days. Samples were analyzed by terminal restriction fragment length polymorphism (T-RFLP) as a culture independent technique that allows for the analysis of microbial communities by generating a fingerprint of the dominant microbial groups (Karlsson et al. 2009). Although, culture-dependent methods have provided information about intestinal microbiota, only about 20% are actually cultivable (Eckburg et al. 2005). Molecular techniques have now become the standard for analyzing the human microbiome (Heilig et al., 2002)

With T-RFLP analysis, the change in microbial community composition was analyzed statistically by non-metric multidimensional scaling (NMDS). Results from NMDS show a trend in which the microbial community composition begins to change after 7 days of yogurt consumption. By days 14 and 42, the microbial communities begin to cluster together for individuals consuming yogurt. This clustering suggests that the microbial communities are becoming structurally more similar after 14 days of yogurt consumption and maintain that similarity for the remainder of the study. Previous research by David et al. (2014) has shown similar short-term alterations of the gut microbiome when a diet is changed between an animal or plant based diet. Furthermore, clustering of GI microbial communities may be the result of the test subjects sharing similar dietary habits, in terms of yogurt consumption. One study analyzing the gut microbiome of individuals fed the same diet showed no clustering of microbial communities, but did see inter-subject variation (Wang et al. 2006). However, it should

be noted that the study by Wang and coworkers only lasted for 10 days, where our study lasted 42 days, allowing for a better representation of the long-term effects an altered-diet might have.

The Shannon-Wiener index, a commonly used estimate of diversity, was used to quantify changes in diversity after the consumption of yogurt (Spellerberg and Fedor, 2003). The Shannon index takes into account both the number of bacterial groups (richness) and the abundance (evenness) of each. The Shannon index also takes into account of changes in rare groups that maybe present within an ecosystem (Karlsson et al. 2010).

Shannon index values based on terminal restriction fragments (T-RFs), showed an increase in microbial diversity for all test subjects that participated in this study. Interestingly, after two weeks of yogurt consumption, the GI diversity appears to be in a state of flux through the duration of the study. This flux could be a result of bacterial species competition with the introduction of probiotic bacteria. Furthermore, individuals with the lowest Day 0 microbial index values (Subjects F and G), had the highest increase in microbial diversity. In comparison, test subject C, which had the highest microbial diversity, did not see as drastic an increase in diversity. Because of individuals G and F having lower diversity it may have provided an opportunity for other microorganism to establish in the GI tract.

Due to budget restraints, clone libraries of only test subjects B, C, and D (control) were sequenced via Sanger method at Days 0 and 42. Results from this study showed a decreased number of cloned sequences for *B. pseudocatenulatum* and *B. bifidum* for test subjects consuming yogurt, while the control subject showed an increase in *B.*

pseudocatenulatum and *B. bifidum*. *Lactobacillus* showed no major trends for the experimental group and control in this study. This could be due to the low number of clones that were Sanger sequenced. Because of this lack of “deep sequencing” there is poor representation of the microbial communities present within the GI tract (Mardis, 2008).

Conclusion

This is the first study to look at the long-scale effects of healthy human test subjects consuming yogurt daily. Many studies utilizing yogurt typically last 10-20 days and use either culturing techniques or identified certain microorganisms using probe markers (Sullivan et al., 2003; Uyeno et al., 2008). Microbial community composition, based on T-RFLP analysis, demonstrates temporal changes in diversity with the consumption of yogurt. Furthermore, individuals with lower microbial GI diversity appear to have the greatest increase in diversity when introduced to yogurt and maintain increased diversity throughout the study. Finally quantitative PCR (qPCR) analysis showed increase in absolute quantity of *Lactobacillus* for test subjects consuming yogurt daily. However, individuals with already diverse communities do not see as drastic a change in *Lactobacillus* gene content. Therefore, this study suggests that regular consumption of yogurt may increase microbial diversity in individuals who have relatively low diversity. Lower microbial diversity of the GI tract has been shown to be associated with recurring CDAD, obesity, and Crohn’s disease, as well as many other diseases (Cheng et al., 2008; Turnbaugh et al., 2009; Ott et al., 2004). Thus, probiotics such as yogurt may provide a therapeutic/preventive approach to these diseases.

Chapter 3: The Effect of Probiotics on Healthy Human GI Microbial Community and Stress Response

Introduction

The fields of microbiology and neuroscience have for the most part developed as separate entities. However, it has recently been recognized that the microbiota within the gut influence multiple aspects of physiology, one such influence is via the gut-brain axis. The term gut-brain axis refers to the bi-directional signaling that exists between the central nervous system (CNS) and the GI tract (Chen et al. 2013, Grenham et al. 2011). Furthermore, it has been suggested that the endocrine (cortisol), immune (cytokines), and neural (vagus and enteric nervous system) pathways are potentially direct and indirect ways in which the gut microbiota can modulate the gut-brain axis (see fig. 2) (Cryan and Dinan, 2012).

Hypothalamic-Pituitary Adrenal (HPA) Axis (Fig. 3-1)

The hypothalamic-pituitary adrenal (HPA) axis is a major part of the neuroendocrine system that is responsible for the regulating stress response, digestion, immune response, as well as many other biological processes (Hans, 1974). In response to a stressor, (i.e. illness or fear) corticotrophin releasing hormone (CRH), also known as corticotrophin releasing factor (CRF), is released from the hypothalamus to the adrenal portion of the pituitary gland. The binding of CRH to receptors on the pituitary gland induces the release of adrenal corticotrophic hormone (ACTH), which is then carried to the adrenal cortex via blood cells. The adrenal gland then releases the corticosteroid, cortisol, into the body to respond to a stressor (Smith and Vale, 2006).

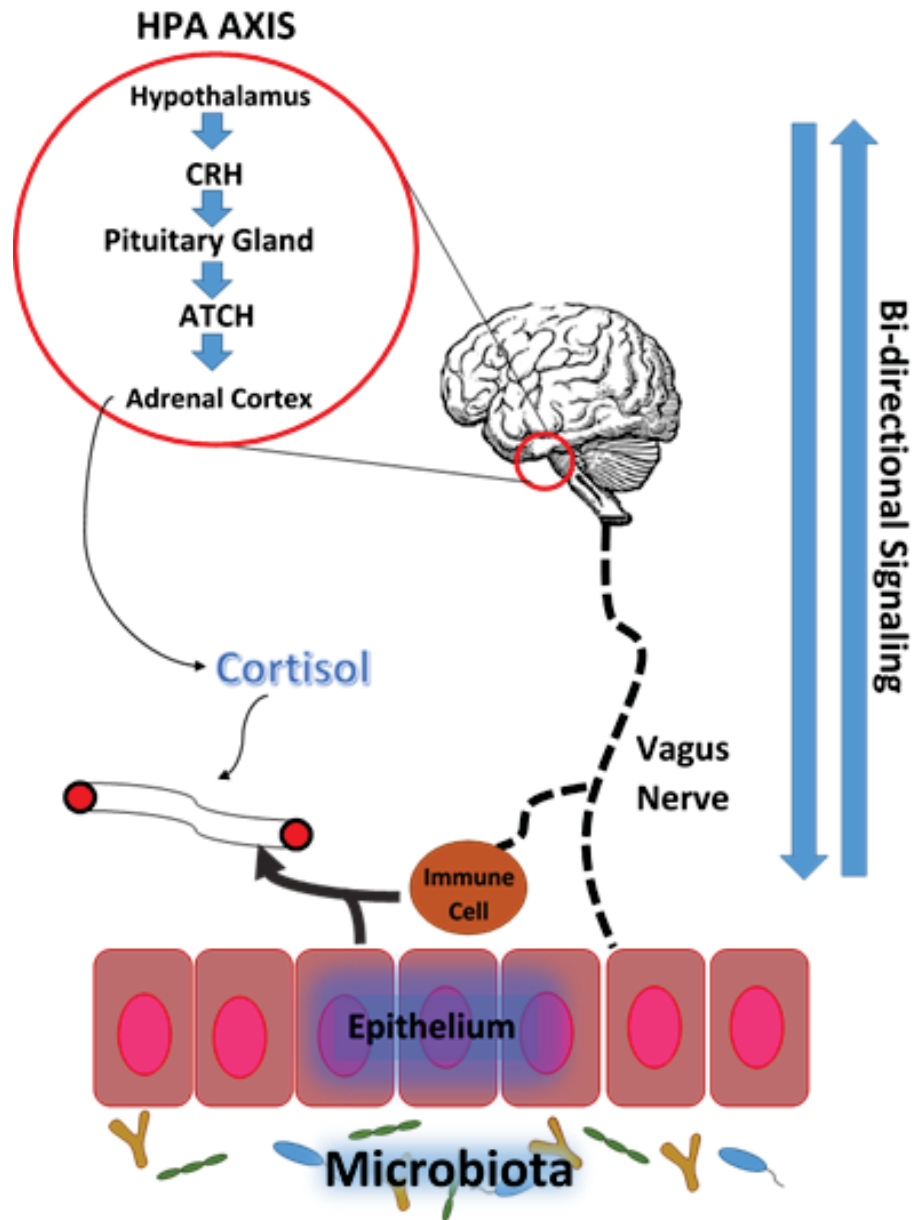


Fig. 3-1 Pathways involved in the bi-directional signaling for the gut-brain axis. The hypothalamic-pituitary-adrenal axis produces cortisol as a result of stress. Cortisol secretions can affect immune cells, such as cytokines; alter gut permeability and change GI microbial composition. GI microbes can also have an influence on brain function via the vagus nerve through tryptophan metabolism and short chain fatty acid (SCFA) production. GI microbiota can also alter immune system by regulating cytokine levels, which can also alter brain function. ACTH, adrenocorticotrophic hormone; CRH, corticotropin-releasing factor. (Modified from Dinan and Cryan, 2013, Chen et al. 2013).

Microbiota and Stress

GF and specific pathogen free (SPF) mice have been the key models for demonstrating the effects microbiota can have on stress. One such study performed by Sudo et al. (2004) was able to show correlation between the HPA axis and gut microbiota using these GF and SPF mice. In this study, GF mice were shown to have an exaggerated corticosterone (CORT) and ACTH response during restraint testing in comparison to SPF mice. Furthermore, re-colonization of GF mice with fecal content of SPF mice was shown to partially reverse the stress response, while monocolonization with *Bifidobacterium infantis* fully reversed the exaggerated HPA response to the restraint stressor, if introduced at earlier developmental stages (Sudo, et al., 2004). Results from these studies suggest that microbial communities within the GI tract, more specifically *B. infantis*, may regulate cortisol production. Further research is required to fully understand which microbes may influence stress response.

A more recent study performed by Heijtz and colleagues (2011) also showed the importance of gut microbiota and their ability to modulate the gut-brain axis. In this study GF mice were shown to have increased motor activity and reduced anxiety-like behaviors in comparison to SPF mice. This behavioral phenotype was also shown to be in relation to an altered expression of genes associated with motor control and anxiety. Similar to Sudo et al. (2004) results, early recolonization of GF mice with fecal material from SPF mice resulted in GF mice displaying similar characteristics to SPF mice (Heijtz et al., 2011). These studies have provided evidence to the role gut microbiota play in the development of healthy CNS function as well as demonstrate their effect on stress and anxiety.

Early Evidence for Psychotropic Benefits from Probiotics

Research on irritable bowel disease (IBD) has led to a link between GI disorders and stress. It has been shown that more than 50%-90% of patients who suffer from IBD also suffer from psychological disturbances during periods of intestinal distress (Messaoudi et al., 2010). Additionally, research has shown that probiotics can alleviate some of the symptoms of GI disorders, which suggests that probiotics may also offer a novel therapeutic approach for the treatment of anxiety and decreasing stress responses (Bercik et al., 2005, Grenham et al., 2011).

Preclinical research using rodent models has provided the interesting possibility of using probiotics for the treatment of CNS disorders (Grenham et al. 2011, Cryan and Dinan, 2012). Work by Gareau and colleagues (2007) has shown that administration of probiotic *Lactobacillus* to neonate maternally separated (MS) rats reduced corticosterone levels after 20 days in comparison to neonate MS rats receiving a saline solution. A recent study analyzing the effects of *Lactobacillus rhamnosus* (JB-1) showed a decreased anxiety-like behavior and reduced levels of corticosterone levels in “normal”, healthy mice (Bravo et al., 2011).

To date, there are very few studies that have analyzed the effects probiotics might have on human test subjects. One study using a combination of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 examined their effects on mental health as well as cortisol levels. This probiotic cocktail had beneficial psychological effects and reduced cortisol levels (Messaoudi et al., 2011).

Although, these studies provide early evidence of probiotics as a therapeutic approach to alleviating psychological and physical stress, there is still not enough information on their effects on human subjects. We will also investigate the effects of this multi-species probiotic on GI microbial community composition. Therefore, this study looks to evaluate the possible beneficial effects probiotics may provide to healthy human test subjects and induced stress through analysis of salivary cortisol. We will also assess the effects multi-species probiotics might have on an individual's perceived stress using a Perceived Stress Questionnaire (PSQ). Based on previous research, I hypothesize introducing probiotics will: 1) increase the abundance of probiotic species in the human GI tract, 2) increase the microbial diversity within the human GI tract, and 3) positively affect stress levels in human test subjects exposed to a stressor.

Methods

Subjects

Fifteen healthy individuals over the age of 18 were included in the study. Individuals who had irregular production of cortisol or were taking any medication that may suppress adrenal function (i.e. anxiety medication or anti-inflammatories) were excluded from this study. Each test subject was provided with a 30-day supply of commercially available probiotic pills containing *Lactobacillus (acidophilus and rhamnosus)* and *Bifidobacterium (lactics, longum, breve, and bifidum)*. All participants were asked to follow their normal diets throughout the entire study. Participants were asked to keep a daily dietary journal. Test subjects were to also report daily exercise, rating the intensity on a scale of 1-4 (1 being of lower intensity and 4 being of higher intensity). Finally, all test subjects were asked to report anytime they felt ill, were

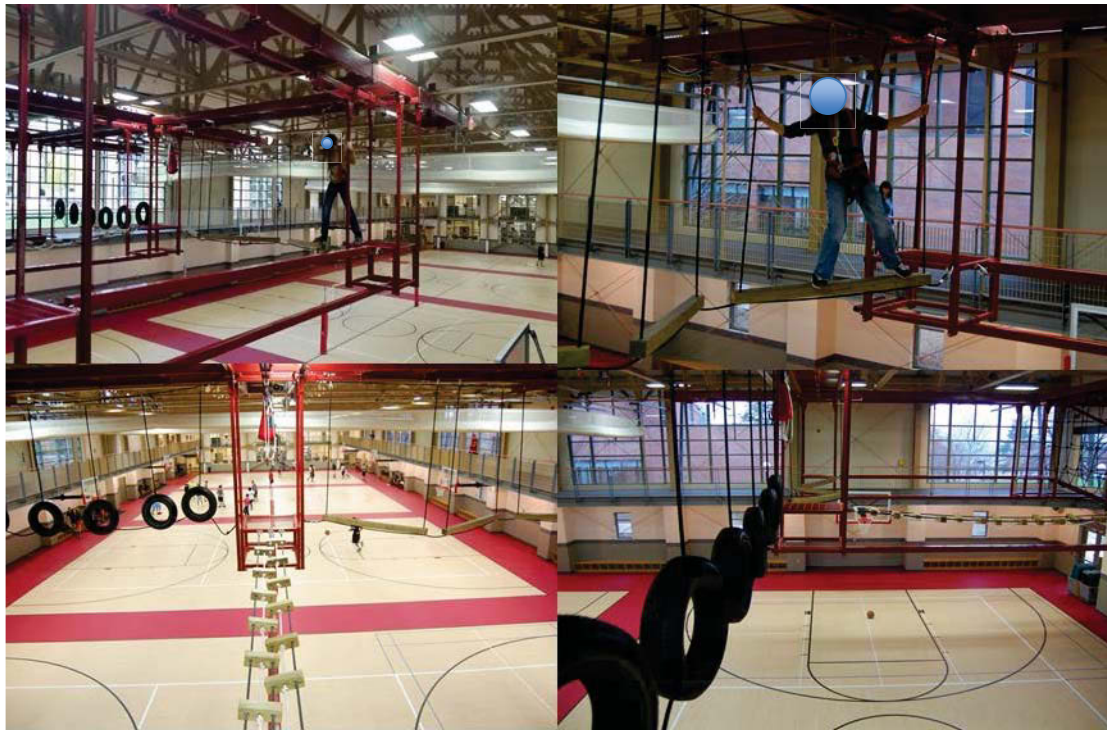
constipated, had diarrhea, or consumed any other form of probiotics or antibiotics. All volunteers gave written informed consent that was approved by the Institutional Review Board (IRB) at Youngstown State University.

Study Design

The total duration of the study, including probiotic pill consumption and follow-up period, was 60 days. Each test subject was required to consume one probiotic pill (~1 billion CFUs per pill) per day for 30 consecutive days. After the 30-day probiotic pill regiment, test subjects refrained from consuming the supplied probiotic pills, but still followed their regular eating habits for 30 additional days.

To induce stress, test subjects were to perform the Elevated High Ropes Course (EHRC) (Fig. 3-2) every two weeks. The high ropes course consisted of various obstacles located 6.09 meters above the ground level. Each test subject was to perform this task to the best of their ability.

Fig. 3-2 Elevated high ropes course (EHRC) to induce stress on human test subject. The course is 4.6 meters up a gymnasium floor. The EHRC is located at Youngstown State University's Recreational Center, Youngstown OH, USA.



Sample Collection

Fresh fecal samples were collected on Day 0 of the probiotic study and every 2 weeks after that until day 30 of the probiotic pill consumption was reached (3 fecal collections; Day 0, Day 15, and Day 30). A final follow-up fecal sample was then collected 30 days after individuals stopped their probiotic regiment (Day 60). Each individual collected his or her own fecal samples in a sterile 90 mL container (Therapak Co., Buford, Georgia USA) and delivered to the laboratory within 12 hours of defecation. Test subjects also collected their own saliva samples in the morning when they first awoke, once before performing EHRC, and once 20 minutes after completing EHRC. Saliva was collected from test subjects using the Salimetrics® Oral Swab (SOS) (Salimetrics, State College, PA USA).

Perceived Stress and Bowel Health Questionnaires

On the same day as fecal collection, a perceived stress questionnaire (PSQ) (Levinstein et al., 1993) was administered consisting of 31 items. The PSQ is an instrument for assessing stressful life events and the circumstances that can trigger disease like symptoms that may alter one psychological state. The questionnaire consisted of 30 questions pertaining to stressful feelings and experiences that individuals may feel during a two-week period and one question pertaining to stressful feelings they experience during the Elevated high ropes course (EHRC). Each test subject was to indicate on a scale from 1 (almost never) to 4 (usually) how frequently they experienced a certain stress-related feeling. Higher scores indicate greater levels of stress.

A Bowel Health Questionnaire was also administered consisting of 11 items. This questionnaire was given to determine the effects probiotic pills might have on various gastrointestinal functions in relation health (i.e. bowel movement, flatulence, etc.) For seven of the questions each test subject was to indicate on a scale from 1 (almost never) to 4 (usually) how frequently they experienced a certain bowel related issues. Higher scores indicate poorer health. Another four question were then asked individuals to record how many times they were constipated, had diarrhea, passed a stool, or have taken a stool softener.

DNA Extraction of Fecal Microbial DNA

Microbial DNA was isolated from 200-300 mg of feces using a modified bead beating method (Yu and Morrison, 2004) to minimize DNA shearing for downstream applications. Extracted DNA was then purified using QIAamp DNA Stool Mini Kit (Qiagen, Valencia, Ca USA) and quantified using a NanoDrop (Thermoscientific, Waltham, MA USA). Extracted microbial DNA was then normalized to 10 ng/ μ l, aliquoted, and stored at -20°C until further analysis.

Microbial DNA Amplification

16s rRNA genes of normalized microbial DNA (gDNA) were amplified using universal bacteria, *Lactobacillus* and *Bifidobacterium* primers (Table 2). Each 20 μ l PCR reaction contained: 0.04 μ l of each primer (0.2u μ M), 10 μ l GoTaq Green Master Mix 1x (Promega, Madison, WI USA), 1 μ l of genomic DNA (10ng/ μ l), and 8.2 μ l of molecular grade water. PCR reactions were amplified using Px2 Thermocycler (Thermoscientific,

Waltham, MA USA) and gel electrophoresis was run to verify successful amplification of PCR products. PCR samples of proper length were then pooled together and shipped to Case Western Reserve University Genomics Core in Cleveland Ohio to perform PCR clean up and Next Generation Sequencing using the MiSeq (Illumina, San Diego, CA USA).

Enzyme Immunoassay of Saliva Samples

To measure salivary cortisol levels an enzyme immunoassay (EIA) was performed on collected saliva samples from test subjects. To perform this task, a High Sensitive Salivary Enzyme Immunoassay Kit (Salimetrics, State College, PA USA) was used according to the manufactures instructions with minor modifications. The EIA samples were read at absorbance 450 nm using a photospectrometer.

Quantitative PCR for Lactobacillus 16s rRNA genes

To determine relative quantity of *Lactobacillus* genes, quantitative PCR (qPCR) was performed using primers Lac1 and Lac2. Standard curves were constructed from isolated *Lactobacillus* DNA from probiotic pills dissolved in autoclaved water and then grown on selective media (LBS). Standards were constructed by performing serial dilutions of 10^{-3} - 10^{-9} of *Lactobacillus* DNA.

Real-time PCR was performed using the iQTM5 real-time detection system (Bio-Rad Inc., Hercules, CA, USA). The qPCR reaction was performed using a total volume of 25 µl of the following reagents: 12.5 µl of SYBR Green buffer ((Qiagen, Valencia, CA, USA), 9.0 µl of RNase free water, 25 µM of primer, and 25 ng of DNA. The reaction

conditions for amplification were 95°C for 3 min, 40 cycles of 95°C for 10s and 55°C for 30s. To determine specificity of amplification, a melt curve was performed after the last cycle, which consisted of 81 cycles by slowing heating from 55°C to 95°C.

Statistical Analyses

To determine cortisol concentrations, a 4-parameter non-linear regression analysis was performed on collected EIA 450 nm absorbance readings. Statistical analysis performed using My Assays Inc., an online database tool (myassays.com).

A two-way repeated measures analysis of variance (ANVOA) was used to determine statistical significance of a probiotic regimen on stress response, *Lactobacillus* relative abundance, and sampling dates. Statistical analysis performed using SPSS 12.0 for windows.

Results

Stress Response for treatment and control groups

Stress response data was collected by subtracting the after cortisol concentrations from the before cortisol concentrations. Calculated stress response was then averaged for each experimental group (control, treatment) at Day 0, 15, 30, and 60. (Table 3-1, Figure 3-1). Two-way repeated measures ANOVA analysis of time of treatment revealed no statistical significance for this study ($p = .585$), but it should be noted that a trend was observed overtime.

Average stress response for the treatment group at Day 0 was 0.228 µg/dL, which was much higher than the control group 0.076 µg/dL. After 15 days of probiotic pill

consumption, stress response decreased to 0.013 µg/dL, which was a lower stress response than the control group (0.072 µg/dL). By Day 30 the stress response increased to 0.062, but day 30 stress response for the treatment group was still lower than Day 0 of the study. After stopping probiotic pill regimen, stress response remained relatively unchanged by Day 60 (0.066) of the study, but still remained lower than Day 0. The control group stress response remained relatively unchanged throughout the entire study, only increasing slightly at Day 60 (0.113).

Quantitative PCR (qPCR) for Lactobacillus 16s rRNA

Average relative abundance for *Lactobacillus* genes was determined for each experimental group in this study (Table 3-2). Two-way repeated measures ANOVA analysis of time and treatment revealed no statistical significance *Lactobacillus* relative abundance for this study ($p = .478$), but it should be noted that a trend was observed overtime.

Relative abundance for *Lactobacillus* 16s rRNA genes was 0.004 at Day 0. At Day 15 of the study a slight increase in relative abundance (0.024) observed and by Day 30 of the study, there was a substantial increase of *Lactobacillus* relative abundance (0.389). Interestingly, after Day 30 of the study probiotic regimen was stopped, this resulted in a decrease in relative abundance to 0.064.

The control group at Day 0 had the lowest relative abundance (0.002) in comparison to all other samples. At Day 15 relative abundance increased to 0.073 and 0.027 at Day 30, but the increase in abundance wasn't as drastic as seen in the treatment group. Relative abundance remained stable from Day 30 to Day 60 (0.019).

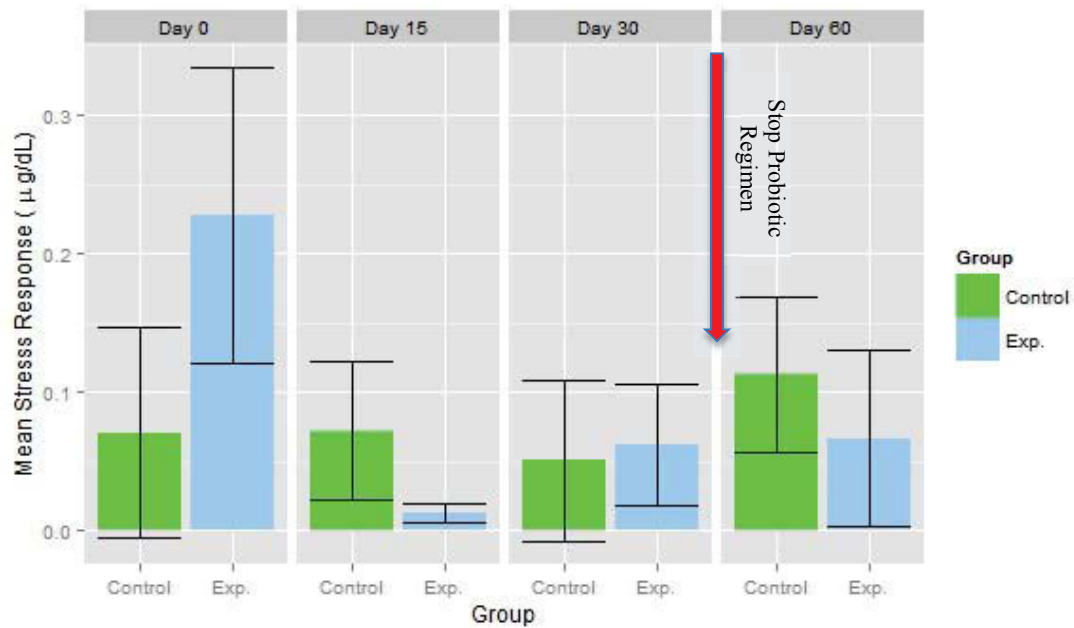


Fig. 3-3 Average change in cortisol (ug/dl) response for control (green) and treatment (blue) over timespan of the study (time shown in days). Treatment was ended at day 30 to create a washout period. Stress Response = after EHC – before EHC.

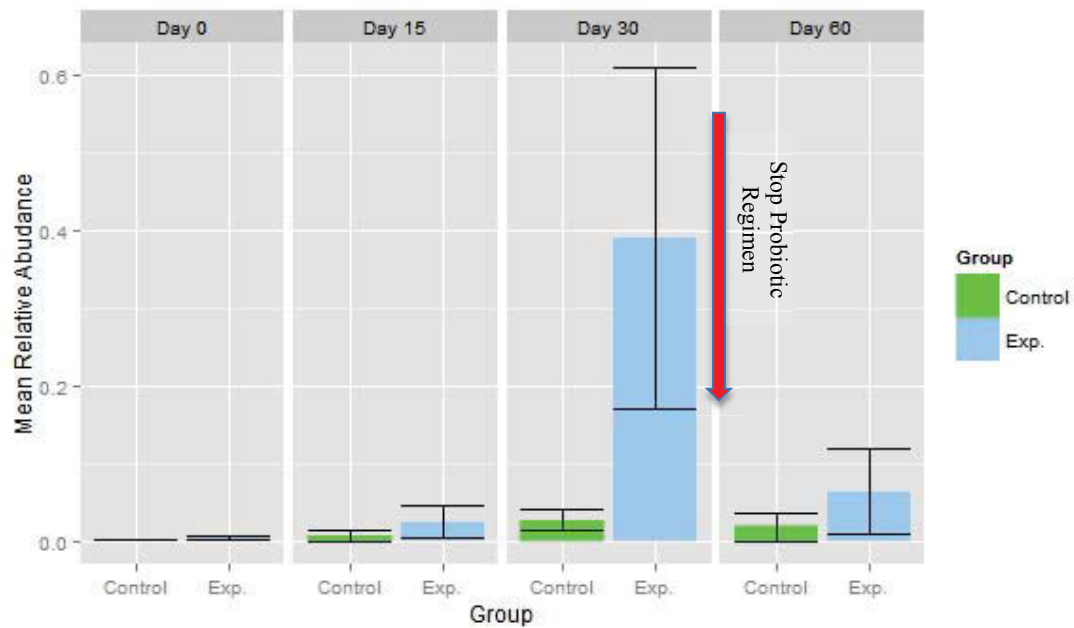


Fig. 3-4 Mean relative abundance of *Lactobacillus* 16S rRNA genes for experimental and control groups. Relative abundance determined by quantitative PCR. Probiotic regimen stopped at day 30 to create a washout period.

Discussion

Many previous studies have used rodent models to analyze the effects microbial communities have on the stress response. However, this study is one of the first to analyze the effects probiotics have on healthy human subjects that are exposed to a stressor. For this study, the stress response was measured by salivary cortisol EIA. This method was selected because it is a proven and reliable method to the amount of unbound cortisol found in blood and allows for stress free analysis (Kirschbaum and Hellhammer, 1994). Molecular analysis via qPCR was also used in this study because it allows for measurement of change in select microbial genes that might be underrepresented in the microbial populations detected using culture techniques and Sanger sequencing.

The calculated stress response from salivary cortisol, although statistically insignificant showed a trend. Individuals that consumed one probiotic pill per day resulted in a drastic decrease in stress response at Day 15 (Fig. 3-3). This decrease in stress response appeared to be maintained throughout the entire study even after the probiotic regimen was stopped. A study using mice found similar decreases in serum cortisol when the mice were fed the probiotic *L. rhamnosus* before being exposed to a stressor (Bravo et al., 2011). Additionally, vagotomized mice that were fed *L. rhamnosus* did not have the same cortisol reduction as mice with the vagal nerve still intact, suggesting the vagus nerve is the major modulatory communication link between gut microbiota and the gut brain axis (Bravo et al., 2011).

QPCR analysis, although statically insignificant, showed an increase in relative abundance for *Lactobacillus* 16s detected rRNA genes from day 0 to Day 15 and a drastic spike of *Lactobacillus* genes by Day 30 (Fig. 3-4). After stopping probiotic treatment on

Day 30, a decrease in relative abundance was seen. In comparison the control group saw a consistent relative abundance of *Lactobacillus* genes throughout the entire study. These results suggest that continued use of probiotic administration results in an increase in *Lactobacillus* over time (Fig. 3-4). However, this increase from probiotic administration is only temporary.

Conclusion

Although, both qPCR and stress response analysis were statically insignificant, a trend was seen in this study that suggests an increase in probiotic bacteria can lead to a decreased stress response for human test subjects. The reason insignificant values were seen could be due to low numbers of test subjects in this study. Ideally, a total of 30 test subjects would have resulted in greater significance. Therefore, future studies should include more test subjects when analyzing the effects probiotics have on stress response.

Chapter IV: Summary, Future Work, and Recommendations

T-RFLP data from Chapter II demonstrated increases in microbial diversity for human test subjects that consumed yogurt for 14 days. Furthermore, test subjects with lower microbial communities saw a more drastic increase in microbial diversity, but test subjects with higher diversity did not see as much increase. After 14 days of yogurt consumption, there is a fluctuation in diversity, which would suggest introducing bacteria from yogurt results in competition for limited space and resources. NMDS analysis of T-RFs showed that microbial communities within the GI tract are influenced by a yogurt

diet. NMDS also suggested that microbial communities of individual subjects become similar in composition over time.

Future work for this study includes analyzing microbial communities by Next Generation Sequencing using primers specific for *Bifidobacteria*, *Lactobacillus*, and universal bacteria. NGS will be used because it provides more reads (96 for Sanger vs 2.5 million for NGS), which will allow for better analysis of the microbial communities that are present. QPCR analysis will also be performed using primers G-Bifid-F and G-Bifid-R to see if *Bifidobacterium* 16s rRNA relative abundance is influenced by yogurt consumption.

Future researchers who wish to analyze the effects on microbial communities should add more test subjects, specifically more controls, to truly understand how microbial communities are affected by yogurt consumption. Finally, to see if microbial communities return to their original state, a washout period after yogurt consumption should be included.

Stress response, determined by cortisol enzyme immunoassay (EIA) from chapter III although statistically insignificant, decreased in healthy human test subject that consumed one probiotic pill per day. Control subjects on the other hand saw no drastic change in their stress response throughout the entire study. QPCR analysis showed an increase in *Lactobacillus* genes at Day 30 for individuals who consumed probiotics. Stopping the probiotic regimen at Day 30 resulted in a drastic decrease in *Lactobacillus* genes. Control subjects showed no drastic changes in *Lactobacillus* genes throughout the entire study.

Future work for the chapter III study includes analyzing microbial communities by NGS using *Bifidobacteria*, *Lactobacillus*, archaeal, Fungal, and universal bacterial. Morning saliva samples will be analyzed using a salivary cortisol EIA to determine the effects of probiotics on daily stress levels. QPCR analysis will be performed using primers G-Bifid-F and G-Bifid-R to see if *Bifidobacterium* 16s rRNA relative abundance is influenced by probiotic administration. Finally, perceived healthy and bowel health questionnaires will be analyzed to determine each individuals daily perceived stress as well as stress when performing EHC. The Bowel Health Questionnaire will be analyzed to describe any results that can be explained to various GI conditions (i.e. stomach flu).

Recommendations for future researchers would be to increase the number of test subjects that participate. Increasing test subjects numbers may result in statistical significance in future work.

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Appendix

I Detailed Protocols for Chapter 2

Powersoil DNA Isolation Kit Protocol

Mo Bio Laboratories, Inc.
Catalog No. 12888-50

Introduction

Powersoil DNA Isolation Kit is a method for isolating genomic DNA from environmental samples that contain high humic acid content including soil, compost, sediment, and manure.

Materials

- 10 µl, 100 µl, and 1000 µl pipettes and tips
- PowerBead tubes (provided)
- Four 2 ml collection tube (provided)
- Spin filter
- Solutions C1, C2, C3, C4, C5, and C6

Method

- 1) Add 0.25 grams of fecal sample to PowerBead tubes and gentle vortex
- 2) Add 60 µl of solution C1 and vortex briefly
 - a. Check solution C1. If solution C1 is precipitated, heat at 60°C until dissolved

- 3) Secure PowerBead tubes horizontally to horizontal vortex adapter and vortex at a maximum speed for 10 minutes
 - a. If using more than 12 preps, increase vortex time by 5-10 minutes
- 4) Centrifuge tubes at 10,000 x g for 30 seconds at room temperature
- 5) Transfer supernatant to clean 2 ml collection tube
 - a. Expect between 400-500 μ l
- 6) Add 250 μ l of solution C2 and vortex for 5 seconds then incubate at 4°C for minutes
- 7) Centrifuge the tubes at room temperature for 1 minute at 10,000 x g
- 8) Avoiding the pellet, transfer up to, but no more than 600 μ l of supernatant to a clean 2 ml collection tube
- 9) Add 200 μ l of solution C3, vortex briefly, and incubate at 4°C for 5 minutes
- 10) Centrifuge the tubes at room temperature for 1 minute at 10,000 x g
- 11) Avoiding the pellet, transfer up to 750 μ l of supernatant into a clean 2 ml collection tube
- 12) **Shake before use!** Add 1200 μ l of solution C4 to the supernatant and vortex for 5 seconds
- 13) Load approximately 675 μ l of supernatant onto a spin filter and centrifuge at 10,000 x g for 1 minute.
- 14) Discard the flow through and add an additional 675 μ l of supernatant to the spin filter and centrifuge at 10,000 x g for 1 minute
- 15) Load remaining supernatant onto spin filter and centrifuge at 10,000 x g for 1 minute

- 16) Add 500 μ l of solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x g
- 17) Discard flow through
- 18) Centrifuge again at room temperature for 10,000 x g at 1 min
- 19) Carefully place spin filter in a clean 2 ml collection tube
 - a. Avoid splashing and C5 solution on spin filter
- 20) Add 100 μ l of solution C6 to center of white membrane filter and centrifuge at room temperature for 30 seconds at 10,000 x g
- 21) Discard spin filter. DNA is now ready for downstream application.

QIAamp® DNA Stool Handbook

Qiagen

Catalog No. 51504

Things to do before starting

- Ensure that Buffer AW1 and Buffer AW2 have been prepared according to the instructions on labels
- Mix all buffers before use
- Prepare 70°C water bath

Materials

- 4 microcentrifuge tubes

Procedure

- 1) Weigh 180-220 mg stool in a 2 ml microcentrifuge tube (not provided) and place on ice
- 2) Add 1.6 ml Buffer ASL to each stool sample and vortex continuously for 1 minute
- 3) Centrifuge sample at full speed for 1 minute to pellet stool particles
- 4) Pipet 1.4 ml of supernatant into a new 2 ml microcentrifuge (not provided)
- 5) Add inhibitEX Tablet to each sample and vortex for 1 minute or until tablet is completely dissolved
- 6) Incubate for 1 minute at room temperature
- 7) Centrifuge at full speed (16,000 x g) for 3 minutes
- 8) Pipet all the supernatant into a new 1.5 ml microcentrifuge tube (not provided) and discard the pellet.
- 9) Centrifuge the sample at full speed for 3 minutes
- 10) Pipet 25 µl proteinase K into a new 2 ml microcentrifuge tube (not provided)

- 11) Add 600 µl of supernatant from step 8 into the 2 ml microcentrifuge tube containing proteinase K
- 12) Add 600 µl Buffer AL and vortex for 15 seconds and incubate at 70°C for 10 minutes
- 13) Add 600 µl of ethanol (96%-100%) to the lysate, and mix by vortexing
- 14) Label the lid of a new QIAamp spin column provided in a 2 ml collection
- 15) Carefully apply 600 µl lysate from step 13 to QIAamp spin column without moistening the rim
- 16) Close the cap and centrifuge at full speed for 1 minute.
- 17) Place the QIAamp spin column in a new 2 ml collection tube, and discard the tube containing filtrate
- 18) Carefully open the QIAamp spin column, apply a second aliquot of 600 µl lysate and centrifuge at full speed for 1 minute.
- 19) Place the QIAamp spin column in a new 2 ml collection tube, and discard the tube containing filtrate
- 20) Repeat steps 18-19 for last lysate
- 21) Carefully open the QIAamp spin column and add 500 µl of Buffer AW2. Close the cap and centrifuge at full speed for 3 minutes
- 22) Discard the collection tube containing the filtrate
- 23) Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate.
- 24) Centrifuge at full speed for 1 minute

- 25) Transfer the QIAamp spin column into a new, labeled 1.5 ml microcentrifuge tube
(not provided)
- 26) Pipet 200 µl of Buffer AE directly onto the QIAamp membrane .
- 27) Incubate at room temperature for 1 minute with the lid closed
- 28) Centrifuge at full speed for 1 minute to elute DNA

Samples are now ready for downstream application

PCR and Terminal Restriction Fragment Length

Polymorphism (T-RFLP) Protocol

PCR protocol with primers 8F (FAM labeled) and 1492R

Materials

- Primers 8F (FAM) and 1492R
- Pipettes and Tips
- Genomic DNA
- Molecular Grade Water
- Wizard® SV Gel and PCR Clean Up System (Promega)
- Restriction Enzyme Hae III
- 2ml Eppendorf Tubes
- 0.2 mL PCR tubes

Master Mix:

Molecular Grade Water:	9.5 µl	
Go Taq Green Master Mix:	12.5 µl	
8F-FAM :	0.5 µl	(10mM)
1492R:	0.5 µl	(10mM)
DNA	2.0 µl	

Thermocycler Settings:

- 94°C for 3 min
 - 94 °C for 30s
 - 55 °C for 30s
 - 72 °C for 1 min 30s
 - 72 °C for 7 min
- 30 cycles

T-RFLP digestion with Hae III

- 1) Add 125 µl of PCR product to 3.5 µl of Hae III (3.0 µl of restriction enzyme to 100 µl of DNA)
- 2) Incubate at 37°C for 16 hours
- 3) Check for successful digestion by running a gel electrophoresis

- 4) Perform PCR clean up using Wizard® SV Gel and PCR Clean Up System

Wizard® SV Gel and PCR Clean Up

Promega Labs
Catalog No. A9281

Procedure

- 1) Add an equal volume of membrane solution to PCR product
- 2) Insert SV minicolumn into provided collection tube
- 3) Transfer prepared PCR product to the minicolumn assembly and incubate at room temperature for 1 minute
- 4) Centrifuge at 16,000 x g for 1 minute
- 5) Discard the flow through and reinsert Minicolumn into collection tube
- 6) Add 700 µl of Membrane Wash Solution (ethanol added) and centrifuge at 16,000 x g for 5 minutes
- 7) Repeat step 4 with 500 µl of Membrane wash solution and centrifuge at 16,000 x g for 5 minutes
- 8) Empty collection tube and recentrifuge assembly for 1 minute with a new microcentrifuge tube. Keep lid of microcentrifuge tube open
- 9) Carefully transfer Minicolumn to a clean 1.5 mL microcentrifuge tube
- 10) Add 35 µl of nuclease-free water and incubate at room temperature for 1 minute
- 11) Centrifuge at 16,000 x g for 1 minute
- 12) Discard minicolumn sample ready for downstream application

Send digested samples to the Ohio State University Plant Microbe and Genomics Facility (Columbus, OH USA) for fragment analysis using a 3730 DNA Analyzer (Applied Biosystems, Inc. Waltham, MA USA) using a LIZ1200 size standard and minimum peak height of 50 fluorescence units.

Cloning

Precloning

LB Medium

20g of LB broth completed to 1 L of ddwater to a pH = 7 using 5N NaOH

Ampicillin

100 mg of Ampicillin in 1 mL of ddwater (100mg/mL). It can then be stored at -20°C before filter sterilization.

LB-Ampicillin Agar (LB-AMP) (final conc. 0.1 mg/ml)

- 1) Prepare 1L of **LB agar** autoclaved
 - **LB agar** autoclaved
 - o 20g LB broth
 - o 20g Tryptic Soy Agar (TSA)
 - o Complete to 1L with ddwater and adjust pH=7 with 5N NaOH.
 - o Autoclave
 - 2) Cool to 55°C
 - 3) Add 1000 µl of 100mg/mL filter-sterilized ampicillin to 1L of autoclaved LB agar and mix by swirling
 - 4) Pour LB-Ampicillin agar into petri dishes (~25 ml/ 100-mm plate)
- Plates can be stored at 4°C for 1-2 months.

Cloning

Materials:

- Strata Clone PCR Cloning Kit (Agilent Technologies)
 - o Cat. # 240205
- 2 ml microcentrifuge tubes (autoclaved)

Ligation- Day 1

- 1) Prepare the ligation reaction mixture by combining in order the following components:
 - a. 3 µl StrataClone Cloning Buffer
 - b. 2 µl of PCR product (5-50 ng) –or- 2 µl of StrataClone Control Insert
 - c. 1 µl StrataClone Vector Mix amp/kan
- 2) Mix gently by pipetting
- 3) Incubate at room temperature for 5 minutes, then place the reaction on ice
 - a. Reaction may be stored at -20°C for later processing

Transformation-Day 1 or Day 2

- Set up a water bath at 42°C
- Check if pUC18 control is diluted 1:10, if not dilute using molecular grade water

Immediately thaw one tube of StrataClone SoloPack competent cells (found in -80°C freezer) on ice for each sample, for control insert and the pUC 18 control. Thawing takes 1-2 minutes.

Procedure:

- 1) Add 1 µl of cloning reaction of each sample to a tube of thawed competent cells. Mix gently by pipetting 2 times
- 2) Add 1 µl of diluted pUC18 (1:10) to a tube of competent cells. Mix gently by pipetting 2 times
- 3) Add 1 µl of control insert to a tube of competent cells. Mix gently by pipetting 2 times
- 4) Incubate transformation mixture on ice for 20 minutes.
 - a. During this time pre-warm LB medium in 42°C water bath
- 5) Heat shock the transformation mixture at 42°C for 45s
- 6) Incubate transformation mixture on ice for 2 minutes
- 7) Add 250 µl of pre-warmed LB medium to transformation reaction mixture
- 8) Allow transformation to reaction to rest for 1 hour and 20 minutes at 37°C in a shaker at 360 rpms.
 - a. During this time , spread 40 µl of 2% X-gal onto each LB-AMP plate
- 9) Plate out two 10 µl of Control Insert and two 30 µl of pUC18 onto 2% X-gal LB-AMP plates
- 10) Plate out 100 µl onto two plates
- 11) Plate 50 µl onto two plates
 - a. When pipetting <50 µl of transformation mixture, add 50 µl of LB medium to transformation mix, gently vortex.
- 12) Plate 10 µl onto two plates
- 13) Incubate plates at 37°C overnight

Analysis-Day 3

Materials:

- UV'd 96 well plate for 1 hour or use autoclaved 96 well plates.
- Autoclaved tray for multichannel pipettor
- Autoclaved toothpicks

Prepare LB AMP 10% glycerol:

For 1 well-plate - 40 ml LB Amp 10% glycerol

- 1) In a sterile Falcon tube combine 40 ml of LB broth and 40 µl of filter sterilized ampicillin, vortex and transfer 36 ml of this solution to another 36 ml sterile Falcon tube.
- 2) Add 4 ml of glycerol to 36 ml of LB AMP broth and vortex (this makes LB AMP 10% glycerol)
- 3) Fill 96 well plate with 150 µl of LB AMP 10% glycerol

Inoculating LB AMP 10% glycerol with colonies (not from Control Insert or pUC 18 control) formed from overnight incubation performed on Day 3

- 1) Pick white colonies with toothpicks and inoculate each well (skip wells A1 and B1).
 - a. Use new toothpick for each colony
 - b. Leave toothpicks in the row just completed as a placeholder, complete the next row and then remove toothpicks from the previously completed column. Repeat until all wells completed
- 2) Place protective film over the plate
- 3) Incubate over night at 37°C while shaking at 360 rpms

The control insert should have >100 colonies per plate. 97% or more should be white colonies. The pUC18 should have > 50 blue colonies.

Confirmation – Day 4

- 1) Remove well plates from incubator
- 2) Using a multichannel pipettor transfer 20 µl onto a new UV sterilized or autoclaved 96 well plate.
- 3) Cover 96 well plate with film and store at -20°C.

Use subsample for PCR confirmation of sample insert

- a. Use 0.5 µl of stock cells of sample for a 25 µl PCR
 - b. Use primers 8F and 1492R for PCR
 - c. Run gel to verify size fragments
- 4) Use film to cover remaining 130 µl and store at 4°C
 - 5) After PCR confirmation, take 50 µl and place it in a autoclave 96 well plate, cover with film and store at -80°C.
 - a. Samples are now ready to be shipped on dry ice for sequencing
 - 6) Cover samples with film and store remaining samples (50 µl) at -80°C

Quantitative PCR

Keep everything on ICE

SYBR Green is light sensitive, keep cold and dark as much as possible.

UV everything beforehand. Sterility is key.

1. Serial Dilutions of Standards
 - a. Serially dilute standard DNA and keep on ice.
 - b. 7 dilutions and a blank of only water will be necessary.
 - c. Dilutions will vary, see example below.
2. Prepare master mix (without SYBR Green)
 - a. Add nuclease free water first
 - b. Add both forward and reverse primers
3. Add DNA to wells of 96 well plate
4. Add SYBR Green to master mix
5. Add master mix to wells of 96 well plate
6. In Dr. Cooper's lab:
 - a. Turn on thermocycler (bottom switch)
 - b. Turn on camera (top switch) [Note: camera takes 10min to warm up]
 - c. Centrifuge 96 well plate up to 600 rpm then stop
7. On the computer:
 - a. Turn on computer if necessary and open program (Bio-Rad)
 - b. Workshop, Add plate, create new
 - c. Flourophore is SYBR Green 1
 - d. Enter experiment name
 - e. Enter standard information (concentration), select units and dilution factor.

It is a good idea to normalize your unknowns to the same concentration as well and depending on your goals (relative quantification versus absolute quantification) it may be necessary.

Standard Serial Dilution example:

For a 1:10 Dilution:

7 tubes, each starts with 18 uL of water.

Add 2 uL of Standard DNA to the first tube (Tube 1) and mix by pipetting.

Take 2 uL from Tube 1 and add to Tube 2 then mix by pipetting.

Take 2 uL from Tube 2 and add to Tube 3 then mix by pipetting.

Continue until serial dilution is complete.

- Standards must be quantified before use and should be normalized to the same concentration.

- Ideally, a tenfold dilution should be used for the serial dilution of standards.
- Standards should be prepared/serially diluted per use to avoid freeze/thaw effects. Standards can be stored and used one or twice.

Plate set up example

STD 1	STD 1	STD 1
STD 2	STD 2	STD 2
STD 3	STD 3	STD 3
STD 4	STD 4	STD 4
STD 5	STD 5	STD 5
STD 6	STD 6	STD 6
STD 7	STD 7	STD 7
BLANK	BLANK	BLANK

- STD are your standards of known concentrations run in triplicate. 1-7 represent the serial dilutions (i.e. STD 1 is diluted 10^{-1} , STD is diluted to 10^{-2} , and so on).
- BLANK is your negative control. This receives water instead of DNA, but is otherwise treated as normal.
- Empty cells are for your samples. Samples should be run in triplicate.

qPCR Reaction set up:

	1 Reaction (25 uL)
SYBR	12.5 uL
Primer 1	0.5 uL
Primer 2	0.5 uL
DNA	2.5 uL
Water	9.0 uL
Total:	25.0 uL

- Multiply all volumes by number of samples (Standards + blanks + unknowns + extra[to account for pipetting error]) to make a master mix.
- When making master mix, DO NOT add DNA. DNA will be added to the wells separately.
- Note in protocol that SYBR green gets added last to the master mix, after DNA has already been added to the wells.
- When adding master mix to wells, add total volume per reaction minus the DNA (i.e. for 25 uL reaction using 2.5 uL DNA per reaction, add 22.5 uL master mix to each well)

**Youngstown State University Institutional Review Board
Informed Consent Form for Research Involving Human Subjects**

Protocol Title: Effects of Yogurt Consumption on Gut Microbial Communities

Principal Investigator: Dr. Carl Johnston

YSU: Biology Department

1. Introduction

You are being asked to take part voluntarily in the research project described below. Please take your time making a decision and feel free to discuss it with your friends and family. Before agreeing to take part in this research study, it is important that you read the consent form that describes the study. Please ask the study researcher or the study staff to explain any words or information that you do not clearly understand.

2. Why is this study being done?

You have been asked to take part in a research study to determine the effect of probiotic pills on the microbial community within the human intestinal tract.

You are one of six subjects participating in this study at YSU.

You are being asked to be in the study because you are an individual in good health, you have no known adverse reactions to probiotics, able to collect your own stool samples, and are willing and capable of following a probiotic/antibiotic free diet for one week followed by a daily consumption of one probiotic pill per day for 30 consecutive days,.

If you decide to enroll in this study, your involvement will last 60 days.

3. What is involved in the study?

If you agree to take part in this study, the research team will:

Provide you with three 150 g (about the size of one yogurt container commonly sold at super markets) servings of yogurt for you to eat each day for 3 weeks.

Provide you with sterile 30 ml wide mouth containers (to avoid leaks) for which you are to collect your stool samples when sample is collected participants will place lid and close the container to avoid contamination. The container will be placed in a double Ziploc bag and kept in a cooler on ice before and during transportation to the laboratory. Hands should be cleaned using soap and water or hand sanitizer. Stray material should be cleaned using sanitary wipes and disinfectant. Any leakage must be cleaned using disinfectant or soap and water. You will provide stool samples to the research team at the start of the study and then once every 15 days for a total of 5 stool samples. Each container with a stool sample will be labeled by the investigator with the date of collection and will be marked with a letter of the alphabet (not with your name) upon receiving.

- Fecal samples must not be in contact with any other surfaces (including hands, toilet paper, wipes, etc) other than the collection sterile cap. If by mistake stools fall into the toilet bowl or feces touch any other surface, those fecal samples cannot be longer used. The participant will have to collect another stool sample in their next bowel movement.

Each human subject also agrees to consume no probiotics (i.e. yogurt, probiotic pills, kefir etc.) for two weeks, followed by consuming one probiotic pill per day for 30 consecutive days

- There is not a typical amount to ingest according to the US dietary guidelines, however, based on other sources of information (e.g. yogurt demand) the average weekly amount of yogurt is 100 g based on national averages on the whole population.

- The amount in the experiment is much more than “average” ingested. Small single portion of yogurt range from 113 g to 150 g and are commonly sold in detachable containers.

4. What are the risks and discomforts of the study?

There are no known risks associated with probiotic pill consumption. Nor is there risk associated with increased risk of developing lactose intolerance. Only individuals with a prior history of regular consumption of probiotics are asked to be in this study. Only individuals trained in microbiology and are able to properly collect their own samples will be asked to participate in this study.

5. What will happen if I am injured in this study?

The Youngstown State University and its affiliates do not offer to pay for or cover the cost of medical treatment for research related illness or injury. No funds have been set aside to pay or reimburse you in the event of such injury or illness. You will not give up any of your legal rights by signing this consent form. You should report any such injury to Carl Johnston (phone 330 941 7002) and to the YSU Institutional Review Board (IRB) at (330-941-2377). If a participant develops i.e. diarrhea he/she should contact his/her physician. Participants can also call Dr. Johnston at (330) 941-7002.

6. Are there benefits to taking part in this study?

There will be no direct benefits to you for taking part in this study. This research may determine what microbes are found in your intestinal tract and if probiotic pills have any effect on the microbial community within your intestinal tract.

7. What other options are there?

You have the option not to take part in this study. There will be no penalties involved if you choose not to take part in this study.

8. Who is paying for this study?

Internal Funding:

Funding to initiate this study is provided by YSU Department of Biological Sciences. Funding has been requested from the YSU College of Graduate Studies and Research.

External funding: N/A

9. What are my costs?

There are no direct costs. You will be responsible for travel to and from the research site and any other incidental expenses.

10. Will I be paid to participate in this study?

You will not be paid for taking part in this research study

11. What if I want to withdraw, or if I am asked to withdraw from this study?

Taking part in this study is voluntary. You have the right to choose not to take part in this study. If you do not take part in the study, there will be no penalty.

If you choose to take part, you have the right to stop at any time. However, we encourage you to talk to a member of the research group so that they know why you are leaving the study. If there are any new findings during the study that may affect whether you want to continue to take part, you will be told about them.

The researcher may decide to stop your participation without your permission, if he thinks your participation in the study may cause you harm. An example of harm would be appearance of symptoms of lactose intolerance.

12. Who do I call if I have questions or problems?

You may ask any questions you have now. If you have questions later, you may call Dr. Carl Johnston (phone: 330-941-7151, email cgjohnston@ysu.edu).

If you have questions or concerns about your participation as a research subject, please contact Dr. Ed Orona, Director of Grants and Sponsored Programs at YSU.

13. What about confidentiality?

1. Your part in this study is confidential. None of the information will identify you by name. All records from samples you provide will be labeled as A, B, C, D, E, or F throughout the experiment. These labels will refer to all data collected from subject A – F.

2. Every effort will be made to keep your information confidential. Your personal information may be disclosed if required by law. Organizations that may inspect and/or copy your research records for quality assurance and data analysis include, but are not necessarily limited to:

The sponsor or an agent for the sponsor
Department of Health and Human Services
YSU Institutional Review Board

Because of the need to release information to these parties, absolute confidentiality cannot be guaranteed. The results of this research study may be presented at meetings or in publications; however, your identity will not be disclosed in those presentations.

All records will be maintained as follows:

Confidentiality documents will be kept locked within Dr. Johnston's office (room 4006 WB) and will be kept separate from all other data collected from this study.

No other documents will mention you by name and you will only have a letter representing data from you. Information associated with you as a subject (designated only by a letter of the alphabet) will be stored in electronic and paper formats and will be released to the public as scientific papers or presentations (with no reference to identity).

3. Participants in this study will be kept confidential with the exception as described above.

14. Mandatory reporting: Not Applicable

15. Authorization Statement

I have read each page of this paper about the study. I know that being in this study is voluntary and I choose to be in this study. I know I can stop being in this study without penalty. I will get a copy of this consent form now and can get information on results of the study later if I wish.

Participant Name: _____

Date: _____

Consent form explained/witnessed by:

Signature: _____

Printed name: _____

Date: _____

Time: _____

II Detailed Protocols for Chapter 3

Salivary Cortisol ELISA

Reagent Preparation:

- 1) Bring all reagents to room temperature
 - a. 1.5 hrs need for 24 mL of assay diluent used in step 5
- 2) Bring microtitre plate to room temperature before use
 - a. Keep the pouch closed until warmed to room temperature
- 3) Prepare a 1x wash buffer by diluting 10x wash buffer with deionized water
 - a. Dilute only enough for current days use
 - i. Discard any leftover reagent
- 4) Prepare a tube with 24 mL of assay diluent for conjugate solution (scale down if necessary).
 - a. Calculate amount of conjugate needed to make 1:1600 dilution (for a 96-well plate add 15µl of the conjugate to 24 ml assay diluent)

DO NOT ADD CONJUGATE TO ASSAY DILUENT UNTIL STEP 3

Procedure:

- 1) Pipette 25µl of standards, controls, and unknowns into appropriate wells of 96-well plate
- 2) Pipette 25µl of assay diluent into zero and non-specific binding (NSB) wells
- 3) Make a 1:1600 dilution of conjugate, mix, and immediately pipette 200µl into each well
- 4) Mix plate by on rotator for 5 minutes at 500 rpm or mix by swirling on table
- 5) Incubate at room temperature for 55 minutes
- 6) Aspirate off solution
- 7) Wash samples by pipetting 300µl of 1x wash buffer into each well.
- 8) Aspirate of solution

- 9) Repeat steps 7-8 three more times
- 10) Add 200 μ l of TMB solution to each well using a multichannel pipette
 - a. May see color change immediately
- 11) Mix on a plate rotator for 5 minutes at 500 rpm or mix by swirling on table
- 12) Incubate the plate in the dark at room temperature for 25 minutes or until good blue color develops.
 - a. Check every 5 minutes
- 13) Add 50 μ l of stop solution to each well with multichannel pipette
- 14) Mix on plate rotator for 3 minutes at 500 rpm or mix by swirling on table
- 15) Read plate at 450 nm within 10 minutes of adding stop solutions

Modified DNA Stool Mini Kit

Reference:

Yu, Z. and M. Morrison. 2004. Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques* 36:808-812.

Reagents (not included in kit):

Tris-HCl (500 mM, pH 8.0): Place 30.28g of Tris in a 500 mL volumetric flask; add ~300 mL milliQ water. Then bring to pH 8.0 by adding concentrated (1M or higher) HCl, then add milliQ water to volume.

EDTA (500 mM, pH 8.0): Place 93.05g of EDTA Disodium Salt Dihydrate in a 500 mL volumetric flask; add ~300 mL milliQ water – heating helps EDTA dissolve; adjust pH close to 8.0 (EDTA only completely dissolves at ~pH 8.0 – when all is dissolved, adjust pH to 8.0, then bring to volume). Autoclave for 15 minutes (if immediately making a solution with this stock, you can use the fresh EDTA solution)

Lysis solution: Place 7.3 g of NaCl and 10 g SDS in an autoclavable bottle; add 25 mL of 500mM Tris-HCl; add 25 mL of 500mM EDTA; add 200mL milliQ water. Stir on a hotplate/stirrer (medium temperature) to dissolve the SDS (this takes a while) – do NOT shake aggressively, this solution foams excessively. Take out the stir bar, then autoclave.

NOTE: This solution separates easily – after autoclaving, a **STERILE** stir bar can be added back. Stir on a medium temperature hotplate before use (solution should be clear)

10M NH₄-Acetate: place 38.54 g Ammonium acetate in a 50 mL volumetric flask; bring up to volume. Mild heating will ease dissolving.

TE buffer: pipet 10mL Tris-HCl (500 mM) and 1mL of 500mM EDTA into a 500mL volumetric flask. Bring to volume, autoclave for 15 minutes.

70% ethanol: bring 70mL of 100% ethanol to 100 mL volume

100% ethanol

Isopropanol

DNase-free RNase: get at the life sciences store-room (Roche catalog #11119915001) in 1 mL tube – good for ~25 extractions. If you know you need large quantities (many extractions) you may want to order directly from Roche – the store room only has a few vials in stock.

Procedure:

Day 1:

Cell Lysis:

Suggestion for fecal samples: do a 105 dry matter on the sample you actually extract.

Turn on the water baths to 37 & 70°C, and let the centrifuge cool to 4°C

To ensure all fluid is incubated properly, you need to spin your tubes in the micro-centrifuge for 2-3 seconds – this is referred to as “quick-spin”

- Weigh up 0.4 g of sterile zirconia/glass beads in 2-mL screw-cap tubes using the porcelain scoop
 - o *0.4g Disruption Beads (RPI 9830)*
- Transfer 0.25 g (or 400ul for *in vitro* samples) of sample into a fresh 2-mL screw-cap tube (acceptable weight range: 0.20 – 0.32 g) – try to keep the rim clean and to not have the sample stick to side of the tube.
- Add 1 mL of lysis solution to the tube and vortex at full speed until beads and sample are reasonably mixed (make sure no sample is sticking to the wall of the tube)
- Homogenize for 3 min at maximum speed with a vortex (tube adapter)
- Incubate at 70°C for 15 min with gentle shaking (inverting) by hand every 5 min
- Centrifuge at 4°C for 5 min at 16,000 X g
- Pipet the **supernatant** into a fresh 2-mL Eppendorf tube
- Add 300 µL of fresh lysis buffer to the screw-cap tube
- Vortex until mixed – make SURE to break up the pellet at the bottom of the tube
- Homogenize for 3 min at maximum speed with a vortex (tube adapter)
- Incubate at 70°C for 15 min with gentle shaking (inverting) by hand every 5 min
- Centrifuge at 4°C for 5 min at 16,000 X g.
- Combine the **supernatant** with the supernatant collected previously

Precipitation of nucleic acids

- Add 260 µL of 10 M ammonium acetate to each tube, vortex to mix.
- Incubate on ice for 5 min
- Centrifuge at 4°C for 10 min at 16,000 X g
- Transfer (split) **supernatant** into two 1.5-mL Eppendorf tubes (use fixed volume, 600 µL will get you most, if not all supernatant)

- Add an equal volume of isopropanol to each eppendorf and mix well
- Incubate on ice for 30 min
- Centrifuge at 4°C for 15 min at 16,000 X g
- Carefully pipet off the **supernatant**, make sure to leave pellet intact!
- Wash the nucleic acids pellet by adding 0.5 mL with 70% ethanol, centrifuge at 4°C for 1 minute at 16,000 X g (to make sure the pellet is intact), then carefully pipet off ethanol – do not damage the pellet!

NOTE: after this step, set the centrifuge to warm up to room temp for the next centrifugation steps

- Dry the pellet in a BSC for 30 mins
- Dissolve the nucleic acid pellet in 100 µL of TE (Tris-EDTA) buffer, this takes some effort – pellet is re-hydrated overnight at 4°C.

Day 2:

- **Pool the two aliquots of dissolved pellet into one tube**

Removal of RNA

- Add 40 µL of DNase-free RNase (final [0.1 µg/µL]). Vortex and quick-spin.
- Incubate at 37°C for 15 min

Part II:

Removal of protein and purification (partial Qiagen Stool mini kit – centrifugation at ROOM TEMPERATURE)

- Add 15 µL of proteinase K, vortex and quick-spin
- Add 200 µL of Buffer AL, vortex and quick-spin
- Incubate at 70°C for 10 min
- Add 200 µL of ethanol, vortex and quick-spin, and pipet all liquid to a *QIAamp* column (don't wet the rim) and centrifuge at 16,000 X g for 1 min at room temp
- Insert columns into a new 2 mL collection tube
- Add 500 µL of Buffer AW1 and centrifuge at 16,000 X g for 1 min, insert columns into new 2 mL collection tube

- Add 500 μ L of Buffer AW2 and centrifuge at 16,000 X g for 1 min, insert columns into new 2 mL collection tube
- Dry the column by centrifugation at RT for 1 min (16,000 X g), insert columns into an appropriately labeled 1.5 mL eppendorf tube
- Add 200 μ L of Buffer AE and incubate at RT for 4 min
- Centrifuge at RT for 1 min to elute DNA and read a 2 μ L sample on the Nano-drop spec
- Freeze samples at -20°C for storage or until further use

Perceived Stress Questionnaire

For each sentence, mark the number that describes how often it applies to you in general, since the last sampling period. Work quickly, without bothering to check your answers.

Almost Never = 1

Sometimes = 2

Often = 3

Usually = 4

1. You feel rested
2. You feel that too many demands are being made on you
3. You are irritable or grouchy
4. You have too many things to do
5. You feel lonely or isolated
6. You find yourself in situations of conflict
7. You feel like you're doing things you really like
8. You feel tired
9. You fear you may not manage to attain your goals
10. You feel calm
11. You have too many decisions to make
12. You feel frustrated
13. You are full of energy
14. You feel tense
15. Your problems seem to be piling up
16. You feel like you're in a hurry
17. You feel safe and protected
18. You have many worries
19. You are under pressure from other people
20. You feel discouraged
21. You enjoy yourself
22. You are afraid for the future
23. You feel you're doing things because you have to not because you want to
24. You feel criticized or judged
25. You are lighthearted
26. You feel mentally exhausted
27. You have trouble relaxing
28. You feel loaded down with responsibility
29. You have enough time for yourself
30. You feel under pressure from deadlines
31. During the stress induction, how stressed did you feel? Rank on a level of 1-10 with one being less stressed and ten being highly stressed.

Bowel Health Questionnaire (BHQ)

For each sentence, mark the number that describes how often it applies to you in general, since the last sampling period. Work quickly, without bothering to check your answers.

Almost Never = 1

Sometimes = 2

Often = 3

Usually = 4

1. During the last 2 weeks, how often have you had discomfort or pain in your abdomen?
2. During the last 2 weeks, how often have you had discomfort or pain in your abdomen after eating?
3. How often in the past 2 weeks have you suffered from feeling an urgency (feeling that you must immediately rush to the toilet to pass a stool)?
4. Have you lost any sleep do to discomfort to abdominal pain or discomfort in the past 2 weeks?
5. In the past two weeks, your stool was very hard?
6. In the last 2 weeks, how often did you have loose, mushy, or watery stools?
7. In the past two weeks, how often have you had flatulence?

For the following questions please write down an estimated amount for each.

1. How often have you had bowel movements in the past 2 weeks?
2. In the past 2 weeks, how often have you been constipated?
3. In the past 2 weeks, how often have you had diarrhea?
4. How many times in the past 2 weeks have you taken laxatives or stool softeners?

**Youngstown State University Institutional Review Board
Informed Consent Form for Research Involving Human Subjects**

Protocol Title: Effects of Probiotics on Human Gut Microbial Community

Composition and Stress

Principal Investigator: Dr. Carl Johnston

YSU: Biology Department

1. Introduction

You are being asked to volunteer in the research project described below. Please take your time making a decision. Feel free to discuss it with your friends and family. Read the consent form describing the study before you agree to take part. Ask the researcher to explain anything that you do not understand.

2. Why is this study being done?

You have been asked to partake in this study to determine the effect probiotic pills have on stress and the microbial community of the human gut.

You are one of 20 subjects in this study.

You are being asked to be in this study because you are:

- Of good health
- Have no known adverse reactions to probiotics (Such as gas or bloating)
- Able to collect your own stool samples
- Willing to take one probiotic pill per day for 30 consecutive days.

If you decide to join in this study, your commitment will last 60 days.

Which includes:

- 30 days of probiotic pill intake
- 30 days normal diet

3. What is involved in the study?

- If you agree to take part in this study, *you will be provided with one pack of probiotic pills (30 pills). You will be required consume the “average” amount of*

one pill per day for 30 consecutive days

While in this study, you should avoid the use of antibiotics. Antibiotics may interfere with results. If a physician prescribes antibiotics, you should take them but report this to the investigator.

Saliva Collection

We will provide you with a sterile Salivettes® to collect a saliva sample.

- *Saliva samples will be collected once before and 20 minutes after elevated high rope course.*

Procedure

- *Remove sterile cotton from plastic tube (salivette)*
- *Place it under your tongue and keep it in your mouth until saturated.*
- *Once saturated, place the cotton back into the plastic tube, cap tightly and label.*

Samples can be kept at room temperature but must be turned into a lab tech on sampling day. Sampling will be at baseline and every 15 days after that for a total of 5 samples over 60 days.

Please refrain from:

- *Eating food or drinking beverages during sample collection.*
- *Drinking coffee 1 hour before collection.*

If you think there is contamination of the salivette® discard it and use a new one.

Fecal Collection

You will provide a fecal sample at the start of this study and then once every 15 days for a total of 5 stool samples.

Provided materials:

- *30 ml sterile wide mouth container*
- *Gloves*
- *Double Ziploc bag*

Collection Procedure

- *Collect fecal samples in 30 ml wide mouth container.*
- *Place lid on container to avoid contamination*
- *Label and date container*
- *Place container in Ziploc bag*
- *Keep sample on ice*
- *Give sample to researcher*

Wash hands after sample collection with soap and water or hand sanitizer.

Stray material or leakage should be cleaned using sanitary wipes and disinfectant.

Samples can no longer be used if:

- They touch any other surfaces (including hands, wipes, etc.) other than the collection tubes.
- By mistake stool falls into the toilet bowl. Collect another stool sample during your next bowel movement.

Fecal and saliva material that touch anywhere other than the interior of the collection tube must be cleaned immediately using sanitary wipes or disinfectant. Sample material could be harmful for other individuals if they come into contact with it.

Dietary/Exercise Log

You are required to keep a daily dietary/exercise log to provide general information of your diet and exercise.

A journal will be provided for you to record:

- *daily food and water consumption.*
- *daily exercise and its intensity*

Make note in the journal if you feel ill, used any antibiotics, or have consumed any other forms of probiotics other than those provided.

Perceived Stress Questionnaire

Every sampling day a survey will be provided to you.

The survey will consist of 42 items:

- *30 questions about daily life stressors*
- *one question on how you felt during elevated high ropes course*
- *10 questions on digestive health*

4. What are the risks and discomforts of the study?

To take part in this study you must have:

- A prior history of probiotic use
- No history of adverse effects (gas and bloating) from probiotics

Talk to a researcher about dropping the study if you experience any adverse effects.

The risks associated with sample collection include:

- Getting saliva or fecal material on your hands.
 - o Some infectious diseases can be transmitted through fecal material and bodily fluids.
 - o Since you will be collecting your own samples, there should be no risk to you as a participant.
 - o Wear gloves and wash your hands after sample collection to avoid contamination.

If you are aware that you have any diseases that may be transmitted in this manner please inform the researcher.

Risk associated with elevated high ropes course:

This study involves inducing stress through elevated high ropes course.

- Individuals with balance issues or a heart problem should not participate in this study.
- Minimal psychological risk associated with this task.

- If you have an extreme fear of heights, this task may be very psychologically stressful
- If you feel you cannot complete the task:
 - stop at that point return to base platform
 - or you may resign from the study

Trained staff will be present at all times to ensure safety, supervise, and to demonstrate. If by an unlikely chance that an adverse event were to occur, there is an emergency medical kit located at the facility and a pulley system is present to lower participants to the ground floor.

5. What will happen if I am injured in this study?

Youngstown State University and its affiliates offer no compensation for research related illness or injury. You will not give up any of your legal rights by signing this consent form.

If you develop any illness or injury (i.e. diarrhea), you should contact one of the following:

- Carl Johnston (phone 330-941-7002)
- YSU Institutional Review Board (IRB) (phone 330-941-2377)
- Your physician.

6. Are there benefits to taking part in this study?

- There will be no direct benefits to you for taking part in this study.
- This research may determine what microbes are found in your intestinal tract

7. What other options are there?

- You have the option not to take part in this study.

- There will be no penalties involved if you choose not to take part in this study.

8. Who is paying for this study?

Internal Funding:

YSU Department of Biological Sciences provides funding for this study. Funding has been requested from the YSU College of Graduate Studies and Research.

External funding: N/A

9. What are my costs?

Travel to and from Youngstown State University, and any other incidental expenses, such as disinfectant or gloves.

10. Will I be paid to participate in this study?

You will not be paid for taking part in this study.

11. What if I want to withdraw, or if I am asked to withdraw from this study?

- Taking part in this study is voluntary.
- You have the right to choose not to take part in this study.
- There will be no penalty if you do not take part in the study.
- You have the right to stop at any time, but we recommend talking with a researcher first.
- You will be told about any new findings that may affect your decision to take part in this study.

The researcher may decide to stop your participation without your permission:

- If he thinks your participation in the study may because you harm.
 - o An example of harm would be appearance of symptoms of lactose intolerance.

12. Who do I call if I have questions or problems?

If you have any questions during the study you may contact Dr. Carl Johnston

- phone: 330-941-7151
- Email: cgjohnston@ysu.edu.

If you have questions or concerns about your participation as a research subject, contact Dr. Ed Orona, Director of Grants and Sponsored Programs at YSU.

13. What about confidentiality?

1. Your part in this study is confidential.
 - a. No information will identify you by name.
 - b. The Principal Investigator (Dr. Johnston) will assign a non-identifying label to you.
 - c. Only Dr. Johnston will have access to the log that links you to your label.
2. This consent form and any confidentiality documents (as mentioned above) will be locked in Dr. Johnston's office. Every effort will be made to keep your information confidential.
3. Your personal information may be disclosed if required by law. Organizations that may inspect and/or copy your research records for quality assurance and data analysis include but are not necessarily limited to:
 - The sponsor or an agent for the sponsor
 - Department of Health and Human Services
 - YSU Institutional Review Board

Absolute confidentiality cannot be guaranteed because of the need to release information to these parties.

The results of this research study may be presented at meetings or in publications. Your identity will not be disclosed in those presentations.

Non-identifying information associated with you as a subject will be stored in electronic and paper formats. This information will be released to the public as scientific papers or presentations.

4. Your name will be kept confidential with the exception as described above.

14. Mandatory reporting: Not Applicable

15. Authorization Statement

- I have read each page of this paper about the study.
- I know that being in this study is voluntary and I choose to be in this study.
- I know I can stop being in this study without penalty.
- I will get a copy of this consent form now and can get information on results of the study later if I wish.

Participant Name: _____

Date: _____

Consent form explained/witnessed by:

Signature: _____

Printed name: _____

Date: _____ Time: _____

November 7, 2014

Dr. Carl Johnston, Principal Investigator
Mr. Daniel Lisko, Co-investigator
Mr. Justin Waldern, Co-investigator
Department of Biological Sciences
UNIVERSITY

RE: HSRC PROTOCOL NUMBER: 044-2015
PROTOCOL TITLE: The Effects of Probiotics on Human Gut Microbial Community
Composition and Stress

Dear Dr. Johnston and Mr. Lisko and Waldern:

The Human Subjects Research Committee of Youngstown State University has reviewed the above mentioned protocol and your revisions and determined that it fully meets YSU Human Subjects Research Guidelines. Therefore, I am pleased to inform you that your project has been fully approved for one year. You must submit a Continuing Review Form and have your project approved by November 6, 2015, if your project continues beyond one year.

Any changes in your research activity should be promptly reported to the Human Subjects Research Committee and may not be initiated without HSRC approval except where necessary to eliminate hazard to human subjects. Any unanticipated problems involving risks to subjects should also be promptly reported to the Human Subjects Research Committee.

Sincerely,

Dr. Scott C. Martin
Interim Associate Dean for Research
Authorized Institutional Official

SCM:cc

c: Dr. Gary Walker, Chair
Department of Biological Sciences

